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<b>(21) International Application Number:</b> PCT/US95/03419 <b>(22) International Filing Date:</b> 17 March 1995 (17.03.95) <b>(30) Priority Data:</b> 08/210,226 18 March 1994 (18.03.94) US <b>(60) Parent Application or Grant</b> <b>(63) Related by Continuation</b> US 08/210,226 (CIP) Filed on 18 March 1994 (18.03.94) <b>(71) Applicants (for all designated States except US):</b> THE GENERAL HOSPITAL CORPORATION [US/US]; 55 Fruit Street, Boston, MA 02114 (US). BOARD OF TRUSTEES OF THE LELAND STANFORD JUNIOR UNIVERSITY [US/US]; 900 Welch Road, Palo Alto, CA 94304 (US). <b>(72) Inventors; and</b> <b>(75) Inventors/Applicants (for US only):</b> AUSUBEL, Frederick [US/US]; 271 Lake Avenue, Newton, MA 02161 (US). DAVID, Ronald, W. [US/US]; 433 Kingsley Avenue, Palo Alto, CA 94301 (US). PREUSS, Daphne [US/US]; 767 Comet Drive, Foster City, CA 94404 (US).	<b>(74) Agent:</b> CLARK, Paul, T.; Fish & Richardson P.C., 225 Franklin Street, Boston, MA 02110 (US).  <b>(81) Designated States:</b> AU, CA, JP, US, European patent (AT, BE, CH, DE, DK, ES, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE).  <b>Published</b> <i>With international search report.</i>	
<b>(54) Title:</b> CLEAVED AMPLIFIED RFLP DETECTION METHODS  <b>(57) Abstract</b>  The invention features methods for generating and detecting polymorphic restriction sites in nucleic acids, and kits for carrying out these methods.		

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CLEAVED AMPLIFIED RFLP DETECTION METHODSBackground of the Invention

5           This invention relates to the generation and detection of genetic polymorphisms.

Genetic maps consisting primarily of restriction fragment length polymorphic (RFLP) markers are being constructed for many organisms, including man.

10       Traditional approaches for detecting RFLPs involve Southern blot hybridization. Recently, techniques based on the polymerase chain reaction (PCR; Mullis et al., Methods Enzymol. 155:350-355, 1987) have been used in addition to, or in place of, traditional RFLP markers in  
15       genetic analysis (Cox et al., BioEssays 13:193-198, 1991). In contrast to traditional RFLP markers, PCR-generated markers can be scored using a small sample of DNA, without the use of radioactivity, and without the need for time-consuming DNA blotting procedures.

20           One widely used PCR-based approach involves the use of single short PCR primers of arbitrary sequence called RAPD primers (for random amplified polymorphic DNA; Reiter et al., Proc. Natl. Acad. Sci. USA 89:1477-1481, 1992; Williams et al., Theoret. Appl. Genet.  
25       82:489-498, 1991). A second category of PCR-based markers are called SSLPs (for simple sequence length polymorphism). The method employing SSLPs is based on amplification across tandem repeats of one or a few nucleotides known as "microsatellites." Microsatellites  
30       occur frequently and randomly in most eukaryotic genomes and display a high degree of polymorphism due to variation in the numbers of repeated units.

A third category of PCR-based markers are called AFLPs (for amplified fragment length polymorphisms). In  
35       the method employing these markers, DNA from two

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polymorphic strains are cleaved with one or two restriction endonucleases, and adapters are ligated to the ends of the cleaved fragments. The fragments are then amplified using primers complementary to the adapter(s). The primers contain short stretches of random nucleotides at their 3' ends, which result in limiting the number of amplified fragments generated.

#### Summary of the Invention

We have developed novel PCR-based methods for detecting the presence or absence of a polymorphic restriction site in a nucleic acid involving the use of differentially labeled PCR primers and oligonucleotides.

Accordingly, in one aspect, the invention features a method for detecting the presence or absence of a polymorphic restriction site in a nucleic acid, involving the steps of (a) amplifying the nucleic acid by PCR using a first and a second primer flanking the polymorphic restriction site, the first primer being tagged with the first member of a specific binding pair, the second primer being tagged with a detectable label; (b) digesting the PCR product of step (a) with the restriction endonuclease corresponding to the polymorphic restriction site; (c) contacting the reaction product of step (b) with the second member of the specific binding pair, immobilized on a solid support; and (d) measuring the level of the detectable label bound to the solid support, the presence of the detectable label bound to the solid support being an indication of the absence of the polymorphic restriction site in the nucleic acid.

In a second aspect, the invention features a method for detecting the presence or absence of a polymorphic restriction site in a nucleic acid, involving the steps of: (a) amplifying the nucleic acid by PCR



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using a first and a second primer flanking the polymorphic restriction site; the first primer being tagged with the first member of a specific binding pair, the second primer being tagged with a first detectable  
5 label; (b) digesting the PCR product of step (a) with the restriction endonuclease corresponding to the polymorphic restriction site; (c) annealing and ligating to the single-stranded ends generated in the reaction of step (b) an oligonucleotide tagged with a second detectable  
10 label; (d) contacting the reaction product of step (c) with the second member of the specific binding pair, immobilized on a solid support; and (e) determining the levels of the first and second detectable labels bound to the solid support, the presence of only the first  
15 detectable label bound to the solid support being an indication of a homozygote lacking the polymorphic restriction site, the presence of only the second detectable label bound to the solid support being an indication of a homozygote containing the polymorphic  
20 restriction site, and the presence of both the first and second detectable labels bound to the solid support being an indication of a heterozygote.

In a third aspect, the invention features a method for detecting the presence or absence of a polymorphic  
25 restriction site in a nucleic acid, the method involving the steps of: (a) amplifying the nucleic acid by PCR using a first and a second primer flanking the polymorphic restriction site, the first primer being tagged with a detectable label, the second primer being  
30 unlabeled; (b) digesting a portion of the reaction of step (a) with the restriction endonuclease corresponding to the polymorphic restriction site, while leaving another portion of the reaction of step (a) undigested; (c) denaturing the digested and undigested portions from  
35 step (b); (d) contacting the product of step (c) with an

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oligonucleotide complementary to a sequence in the strand of the product of step (c) containing the detectable label, the sequence being between the polymorphic restriction and the sequence complementary to the second  
5 primer, the oligonucleotide being tagged with a first member of a specific binding pair; (e) contacting the reaction product of step (d) with the second member of the specific binding pair, immobilized on a solid support; and (f) determining the ratio of the levels of  
10 the detectable label bound to the solid support between undigested and digested samples, a ratio of 1:0 between equivalent portions of the undigested and digested samples being an indication of a homozygote containing the polymorphic restriction site, a ratio of 1:1 between  
15 equivalent portions of the undigested and digested samples being an indication of a homozygote lacking the polymorphic restriction site, and a ratio of 2:1 between equivalent portions of the undigested and digested samples being an indication of a heterozygote.

20 In a fourth aspect, the invention features a method for detecting the presence or absence of a polymorphic restriction site in a nucleic acid, involving the steps of: (a) amplifying the nucleic acid by PCR using a first and a second primer flanking the  
25 polymorphic restriction site, the first primer being tagged with a first detectable label, the second primer being tagged with a second detectable label; (b) digesting the reaction product of step (a) with the restriction endonuclease corresponding to the polymorphic  
30 restriction site; (c) denaturing the reaction product of step (b); (d) contacting the product of step (c) with a first and a second oligonucleotide, the first oligonucleotide being complementary to a first sequence in the strand of the product of step (c) containing the  
35 first detectable label, the first sequence being between

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the polymorphic restriction site and the sequence corresponding to the first primer, the first oligonucleotide being tagged with the first member of a first specific binding pair, the second oligonucleotide  
5 being complementary to a second sequence in the strand of the product of step (c) containing the second detectable label, the second sequence being on the same side of the polymorphic restriction site as the first sequence, the second sequence not being contained within or being  
10 complementary to either of the first or second primers, the second oligonucleotide being tagged with the first member of a second specific binding pair; (e) contacting a first portion of the reaction product of step (d) with the second member of the first specific binding pair,  
15 immobilized on a first solid support; (f) contacting a second portion of the reaction product of step (d) with the second member of the second specific binding pair, immobilized on a second solid support; and (g) determining the ratio of the levels of the first and  
20 second detectable labels bound to the first and second solid supports, a ratio of 1:0 between equivalent amounts of the first and second portions being an indication of a homozygote containing the polymorphic restriction site, a ratio of 1:1 between equivalent amounts of the first and  
25 second portions being an indication of a homozygote lacking the polymorphic restriction site, and a ratio of 2:1 between equivalent amounts of the first and second portions being an indication of a heterozygote.

In a fifth aspect, the invention features a method  
30 for detecting the presence or absence of a polymorphic restriction site in a nucleic acid, involving the steps of: (a) amplifying the nucleic acid by PCR using a first and a second primer flanking the polymorphic restriction site, the first primer being tagged with a first  
35 detectable label, the second primer being tagged with a

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second detectable label; (b) digesting the reaction product of step (a) with the restriction endonuclease corresponding to the polymorphic restriction site; (c) denaturing the reaction product of step (b); (d)

5 contacting the product of step (c) with a first and a second oligonucleotide, the first oligonucleotide being complementary to a first sequence in the strand of the product of step (c) containing the first detectable label, the first sequence being between the polymorphic

10 restriction site and the sequence complementary to the second primer, the first oligonucleotide being tagged with the first member of a first specific binding pair, the second oligonucleotide being complementary to a second sequence in the strand of the product of step (c)

15 containing the second detectable label, the second sequence being on the same side of the polymorphic restriction site as the first sequence, the second sequence not being contained within or being complementary to either of the first or second primers,

20 the second oligonucleotide being tagged with the first member of a second specific binding pair; (e) contacting a first portion of the reaction product of step (d) with the second member of the first specific binding pair, immobilized on a first solid support; (f) contacting a

25 second portion of the reaction product of step (d) with the second member of the second specific binding pair, immobilized on a second solid support; and (g) determining the ratio of the levels of the first and second detectable labels bound to the first and second

30 solid supports, a ratio of 0:1 between equivalent amounts of the first and second portions being an indication of a homozygote containing the polymorphic restriction site, a ratio of 1:1 between equivalent amounts of the first and second portions being an indication of a homozygote

35 lacking the polymorphic restriction site, and a ratio of

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1:2 between equivalent amounts of the first and second portions being an indication of a heterozygote.

In a sixth aspect, the invention method for detecting the presence or absence of a polymorphic restriction site in a nucleic acid, involving the steps of: (a) amplifying the nucleic acid by PCR using a first and a second primer flanking the polymorphic restriction site, the first primer being tagged with a first detectable label, the second primer being tagged with a second detectable label; (b) digesting the reaction product of step (a) with the restriction endonuclease corresponding to the polymorphic restriction site; (c) denaturing the reaction product of step (b); (d) contacting the product of step (c) with a first and a second oligonucleotide, the first oligonucleotide being complementary to a first sequence in the strand of the product of step (c) containing the first detectable label, the first sequence being between the polymorphic restriction site and the sequence corresponding to the first primer, the first oligonucleotide being tagged with the first member of a specific binding pair, the second oligonucleotide being complementary to a second sequence in the strand of the product of step (c) containing the second detectable label, the second sequence being on the same side of the polymorphic restriction site as the first sequence, the second sequence not being contained within or being complementary to either of the first or second primers, the second oligonucleotide being tagged with the first member of the specific binding pair; (e) contacting the reaction product of step (d) with the second member of the specific binding pair, immobilized on a solid support; and (f) determining the ratio of the levels of the first and second detectable labels bound to the solid support, a ratio of 1:0 being an indication of a homozygote containing the polymorphic restriction site,

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a ratio of 1:1 being an indication of a homozygote lacking the polymorphic restriction site, and a ratio of 2:1 being an indication of a heterozygote.

In a seventh aspect, the invention features a  
5 method for detecting the presence or absence of a  
polymorphic restriction site in a nucleic acid, involving  
the steps of: (a) amplifying the nucleic acid by PCR  
using a first and a second primer flanking the  
polymorphic restriction site, the first primer being  
10 tagged with a first detectable label, the second primer  
being tagged with a second detectable label; (b)  
digesting the reaction product of step (a) with the  
restriction endonuclease corresponding to the polymorphic  
restriction site; (c) denaturing the reaction product of  
15 step (b); (d) contacting the product of step (c) with a  
first and a second oligonucleotide, the first  
oligonucleotide being complementary to a first sequence  
in the strand of the product of step (c) containing the  
first detectable label, the first sequence being between  
20 the polymorphic restriction site and the sequence  
complementary to the second primer, the first  
oligonucleotide being tagged with the first member of a  
specific binding pair, the second oligonucleotide being  
complementary to a second sequence in the strand of the  
25 product of step (c) containing the second detectable  
label, the second sequence being on the same side of the  
polymorphic restriction site as the first sequence, the  
second sequence not being contained within or being  
complementary to either of the first or second primers,  
30 the second oligonucleotide being tagged with the first  
member of the specific binding pair; (e) contacting the  
reaction product of step (d) with the second member of  
the specific binding pair, immobilized on a solid  
support; and (f) determining the ratio of the levels of  
35 the first and second detectable labels bound to the solid

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support, a ratio of 0:1 being an indication of a homozygote containing the polymorphic restriction site, a ratio of 1:1 being an indication of a homozygote lacking the polymorphic restriction site, and a ratio of 1:2  
5 being an indication of a heterozygote.

In an eighth aspect, the invention features a method for detecting the presence or absence of a polymorphic restriction site in a nucleic acid, involving the steps of: (a) amplifying the nucleic acid by PCR  
10 using a first and a second primer flanking the polymorphic restriction site, the first primer being tagged with the first member of a first specific binding pair, the second primer being tagged with a detectable label; (b) digesting the reaction product of step (a)  
15 with the restriction endonuclease corresponding to the polymorphic restriction site; (c) contacting the reaction product of step (b) with the second member of the first specific binding pair, immobilized on a first solid support; (d) denaturing the reaction product of step (c)  
20 not bound to the first solid support; (e) contacting the product of step (d) with an oligonucleotide complementary to a sequence in the strand of the product of step (d) containing the detectable label, the sequence being between the polymorphic restriction site and the sequence  
25 corresponding to the second primer, the oligonucleotide being tagged with the first member of a second specific binding pair; (f) contacting the reaction product of step (e) with the second member of the second specific binding pair, immobilized on a second solid support; and (g)  
30 determining the ratio of the level of the detectable label bound to the first solid support to the level of the detectable label bound to the second solid support, a ratio of 0:1 being an indication of a homozygote containing the polymorphic restriction site, in a case  
35 where the total amount of the reaction product from step

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(c) not bound to the first solid support was used in steps (d), (e), and (f); a ratio of 1:0 being an indication of a homozygote lacking the polymorphic restriction site, in a case where the total amount of the reaction product from step (c) not bound to the first solid support was used in steps (d), (e), and (f); and a ratio of 1:1 being an indication of a heterozygote, in a case where the total amount of the reaction product from step (c) not bound to the first solid support was used in steps (d), (e), and (f). In a ninth aspect, the invention features a method for detecting the presence or absence of a polymorphic restriction site in a nucleic acid, involving the steps of: (a) amplifying the nucleic acid by PCR using a first and a second primer flanking the polymorphic restriction site, the first primer being tagged with a detectable label, the second primer being unlabeled; (b) digesting the reaction product of step (a) with the restriction endonuclease corresponding to the polymorphic restriction site; (c) annealing and ligating to the single-stranded ends generated in the reaction of step (b) a first oligonucleotide tagged with the first member of a first specific binding pair; (d) contacting the reaction product of step (c) with the second member of the first specific binding pair, immobilized on a first solid support; (e) denaturing the reaction product of step (d) not bound to the first solid support; (f) contacting the product of step (e) with a second oligonucleotide complementary to a sequence in the strand of the product of step (e) containing the detectable label, the sequence being between the polymorphic restriction site and either the sequence corresponding to the first primer or the sequence complementary to the second primer, the second oligonucleotide being tagged with the first member of a second specific binding pair; (g) contacting the reaction product of step (f) with the



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second member of the second specific binding pair,  
immobilized on a second solid support; and (h)  
determining the ratio of the level of the detectable  
label bound to the first solid support to the level of  
5 the detectable label bound to the second solid support, a  
ratio of 1:0 being an indication of a homozygote  
containing the polymorphic restriction site, in a case  
where the total amount of the reaction product from step  
(d) not bound to the first solid support was used in  
10 steps (e), (f), and (g); a ratio of 0:1 being an  
indication of a homozygote lacking the polymorphic  
restriction site, in a case where the total amount of the  
reaction product from step (d) not bound to the first  
solid support was used in steps (e), (f), and (g); and a  
15 ratio of 1:1 being an indication of a heterozygote; in a  
case where the total amount of the reaction product from  
step (d) not bound to the first solid support was used in  
steps (e), (f), and (g).

In a tenth aspect, the invention features a method  
20 for detecting the presence or absence of a polymorphic  
restriction site in a nucleic acid, involving the steps  
of: (a) amplifying the nucleic acid by PCR using a first  
and a second primer flanking the polymorphic restriction  
site, the first primer being tagged with the first member  
25 of a first specific binding pair, the second primer being  
tagged with a detectable label; (b) digesting the  
reaction product of step (a) with the restriction  
endonuclease corresponding to the polymorphic restriction  
site; (c) contacting the reaction product of step (b)  
30 with the second member of the first specific binding  
pair, immobilized on a first solid support; (d)  
denaturing the reaction product from step (c) not bound  
to the first solid support; (e) contacting the product of  
step (d) with an oligonucleotide complementary to a  
35 sequence in the strand of the product of step (d)

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containing the detectable label, the sequence being between the polymorphic restriction site and the sequence corresponding to the second primer, the oligonucleotide being immobilized on a second solid support; and (f)

5 determining the ratio of the level of the detectable label bound to the first solid support to the level of the detectable label bound to the second solid support, a ratio of 0:1 being an indication of a homozygote containing the polymorphic restriction site, in a case

10 where the total amount of the reaction product from step (c) not bound to the first solid support was used in steps (d) and (e); a ratio of 1:0 being an indication of a homozygote lacking the polymorphic restriction site, in a case where the total amount of the reaction product

15 from step (c) not bound to the first solid support was used in steps (d) and (e); and a ratio of 1:1 being an indication of a heterozygote, in a case where the total amount of the reaction product from step (c) not bound to the first solid support was used in steps (d) and (e).

20 In an eleventh aspect, the invention features a method for detecting the presence or absence of a polymorphic restriction site in a nucleic acid, involving the steps of: (a) amplifying the nucleic acid by PCR using a first and a second primer flanking the

25 polymorphic restriction site, the first primer being tagged with a detectable label, the second primer being unlabeled; (b) digesting the reaction product of step (a) with the restriction endonuclease corresponding to the polymorphic restriction site; (c) annealing and ligating

30 to the single-stranded ends generated in the reaction of step (b) a first oligonucleotide tagged with the first member of a first specific binding pair; (d) contacting the reaction product of step (c) with the second member of the first specific binding pair, immobilized on a

35 first solid support; (e) denaturing the reaction product

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of step (d) not bound to the first solid support; (f) contacting the product of step (e) with a second oligonucleotide complementary to a sequence in the strand of the product of step (e) containing the detectable  
5 label, the sequence being between the polymorphic restriction site and either the sequence corresponding to the first primer or the sequence complementary to the second primer, the second oligonucleotide being immobilized on a second solid support; and (g)  
10 determining the ratio of the level of the detectable label bound to the first solid support to the level of the detectable label bound to the second solid support, a ratio of 1:0 being an indication of a homozygote containing the polymorphic restriction site, in a case  
15 where the total amount of the reaction product from step (d) not bound to the first solid support was used in steps (e) and (f); a ratio of 0:1 being an indication of a homozygote lacking the polymorphic restriction site, in a case where the total amount of the reaction product  
20 from step (d) not bound to the first solid support was used in steps (e) and (f); and a ratio of 1:1 being an indication of a heterozygote, in a case where the total amount of the reaction product from step (d) not bound to the first solid support was used in steps (e) and (f).  
25 In a twelfth aspect, the invention features a method for detecting the presence or absence of a polymorphic restriction site in a nucleic acid, involving the steps of: (a) amplifying the nucleic acid by PCR using a first and a second primer flanking the  
30 polymorphic restriction site, the first primer containing a first sequence not complementary to or present in the nucleic acid, the second primer containing a second sequence not complementary to or present in the nucleic acid; (b) amplifying the product of step (a) by PCR using  
35 a third and a fourth primer, the third primer containing

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the first sequence or a sequence complementary to the first sequence, the third primer being tagged with the first member of a specific binding pair, the fourth primer containing the second sequence or a sequence  
5 complementary to the second sequence, the fourth primer being tagged with a detectable label; (c) digesting the product of step (b) with the restriction endonuclease corresponding to the polymorphic restriction site; (d) contacting the reaction product of step (c) with the  
10 second member of the specific binding pair, immobilized on a solid support; and (e) measuring the level of the detectable label bound to the solid support, the presence of the detectable label bound to the solid support being an indication of the absence of the polymorphic  
15 restriction site in the nucleic acid.

In a thirteenth aspect, the invention features a method for detecting the presence or absence of a polymorphic restriction site in a nucleic acid, involving the steps of: (a) amplifying the nucleic acid by PCR  
20 using a first and a second primer flanking the polymorphic restriction site, the first primer containing a first sequence not complementary to or present in the nucleic acid, the second primer containing a second sequence not complementary to or present in the nucleic  
25 acid; (b) amplifying the product of step (a) by PCR using a third and a fourth primer, the third primer containing the first sequence or a sequence complementary to the first sequence, the third primer being tagged with the first member of a specific binding pair, the fourth  
30 primer containing the second sequence or a sequence complementary to the second sequence, the fourth primer being tagged with a detectable label; (c) digesting the PCR product of step (b) with the restriction endonuclease corresponding to the polymorphic restriction site; (d)  
35 annealing and ligating to the single-stranded ends

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generated in the reaction of step (c) an oligonucleotide tagged with a second detectable label; (e) contacting the reaction product of step (d) with the second member of the specific binding pair, immobilized on a solid support; and (f) determining the levels of the first and second detectable labels bound to the solid support, the presence of only the first detectable label bound to the solid support being an indication of a homozygote lacking the polymorphic restriction site, the presence of only the second detectable label bound to the solid support being an indication of a homozygote containing the polymorphic restriction site, and the presence of both the first and second detectable labels bound to the solid support being an indication of a heterozygote.

In a fourteenth aspect, the invention features a method for detecting the presence or absence of a polymorphic restriction site in a nucleic acid, involving the steps of: (a) amplifying the nucleic acid by PCR using a first and a second primer flanking the polymorphic restriction site, the first primer containing a first sequence not complementary to or present in the nucleic acid; (b) amplifying the product of step (a) by PCR using a third primer and the second primer, the third primer containing the first sequence, the third primer being tagged with a detectable label; (c) digesting a portion of the reaction of step (b) with the restriction endonuclease corresponding to the polymorphic restriction site, while leaving another portion of the reaction of step (b) undigested; (d) denaturing the digested and undigested portions from step (c); (e) contacting the product of step (d) with an oligonucleotide complementary to a second sequence in the strand of the product of step (d) containing the detectable label, the second sequence being between the polymorphic restriction site and the sequence complementary to the second primer, the

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oligonucleotide being tagged with a first member of a specific binding pair; (f) contacting the reaction product of step (e) with the second member of the specific binding pair, immobilized on a solid support;  
5 and (g) determining the ratio of the levels of the detectable label bound to the solid support between undigested and digested samples, a ratio of 1:0 between equivalent portions of the undigested and digested samples being an indication of a homozygote containing  
10 the polymorphic restriction site, a ratio of 1:1 between equivalent portions of the undigested and digested samples being an indication of a homozygote lacking the polymorphic restriction site, and a ratio of 2:1 between equivalent portions of the undigested and digested  
15 samples being an indication of a heterozygote.

In a fifteenth aspect, the invention features a method for detecting the presence or absence of a polymorphic restriction site in a nucleic acid, involving the steps of: (a) amplifying the nucleic acid by PCR  
20 using a first and a second primer flanking the polymorphic restriction site, the first primer containing a first sequence not complementary to or present in the nucleic acid, the second primer containing a second sequence not complementary to or present in the nucleic  
25 acid; (b) amplifying the product of step (a) by PCR using a third and a fourth primer, the third primer containing the first sequence or a sequence complementary to the first sequence, the third primer being tagged with a first detectable label, the fourth primer containing the  
30 second sequence or a sequence complementary to the second sequence, the fourth primer being tagged with a second detectable label; (c) digesting the reaction product of step (b) with the restriction endonuclease corresponding to the polymorphic restriction site; (d) denaturing the  
35 reaction product of step (c); (e) contacting the product

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of step (d) with a first and a second oligonucleotide, the first oligonucleotide being complementary to a third sequence in the strand of the product of step (d) containing the first detectable label, the third sequence  
5 being between the polymorphic restriction site and the sequence corresponding to or complementary to the first primer, the first oligonucleotide being tagged with the first member of a first specific binding pair, the second oligonucleotide being complementary to a fourth sequence  
10 in the strand of the product of step (d) containing the second detectable label, the fourth sequence being on the same side of the polymorphic restriction site as the third sequence, the fourth sequence not being contained within or being complementary to any of the primers, the  
15 second oligonucleotide being tagged with the first member of a second specific binding pair; (f) contacting a first portion of the reaction product of step (e) with the second member of the first specific binding pair, immobilized on a first solid support; (g) contacting a  
20 second portion of the reaction product of step (e) with the second member of the second specific binding pair, immobilized on a second solid support; and (h) determining the ratio of the levels of the first and second detectable labels bound to the first and second  
25 solid supports, a ratio of 1:0 between equivalent amounts of the first and second portions being an indication of a homozygote containing the polymorphic restriction site, a ratio of 1:1 between equivalent amounts of the first and second portions being an indication of a homozygote  
30 lacking the polymorphic restriction site, and a ratio of 2:1 between equivalent amounts of the first and second portions being an indication of a heterozygote.

In a sixteenth aspect, the invention features a method for detecting the presence or absence of a  
35 polymorphic restriction site in a nucleic acid, involving

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the steps of: (a) amplifying the nucleic acid by PCR using a first and a second primer flanking the polymorphic restriction site, the first primer containing a first sequence not complementary to or present in the  
5 nucleic acid, the second primer containing a second sequence not complementary to or present in the nucleic acid; (b) amplifying the product of step (a) by PCR using a third and a fourth primer, the third primer containing the first sequence or a sequence complementary to the  
10 first sequence, the third primer being tagged with a first detectable label, the fourth primer containing the second sequence or a sequence complementary to the second sequence, the fourth primer being tagged with a second detectable label; (c) digesting the reaction product of  
15 step (b) with the restriction endonuclease corresponding to the polymorphic restriction site; (d) denaturing the reaction product of step (c); (e) contacting the product of step (d) with a first and a second oligonucleotide, the first oligonucleotide being complementary to a third  
20 sequence in the strand of the product of step (d) containing the first detectable label, the third sequence being between the polymorphic restriction site and the sequence corresponding to or complementary to the second primer, the first oligonucleotide being tagged with the  
25 first member of a first specific binding pair, the second oligonucleotide being complementary to a fourth sequence in the strand of the product of step (d) containing the second detectable label, the fourth sequence being on the same side of the polymorphic restriction site as the  
30 third sequence, the fourth sequence not being contained within or being complementary to any of the primers, the second oligonucleotide being tagged with the first member of a second specific binding pair; (f) contacting a first portion of the reaction product of step (e) with the  
35 second member of the first specific binding pair,



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- immobilized on a first solid support; (g) contacting a second portion of the reaction product of step (e) with the second member of the second specific binding pair, immobilized on a second solid support; and (h)
- 5 determining the ratio of the levels of the first and second detectable labels bound to the first and second solid supports, a ratio of 0:1 between equivalent amounts of the first and second portions being an indication of a homozygote containing the polymorphic restriction site, a
- 10 ratio of 1:1 between equivalent amounts of the first and second portions being an indication of a homozygote lacking the polymorphic restriction site, and a ratio of 1:2 between equivalent amounts of the first and second portions being an indication of a heterozygote.
- 15 In a seventeenth aspect, the invention features a method for detecting the presence or absence of a polymorphic restriction site in a nucleic acid, involving the steps of: (a) amplifying the nucleic acid by PCR using a first and a second primer flanking the
- 20 polymorphic restriction site, the first primer containing a first sequence not complementary to or present in the nucleic acid, the second primer containing a second sequence not complementary to or present in the nucleic acid; (b) amplifying the product of step (a) by PCR using
- 25 a third and a fourth primer, the third primer containing the first sequence or a sequence complementary to the first sequence, the third primer being tagged with a first detectable label, the fourth primer containing the second sequence or a sequence complementary to the second
- 30 sequence, the fourth primer being tagged with a second detectable label; (c) digesting the reaction product of step (b) with the restriction endonuclease corresponding to the polymorphic restriction site; (d) denaturing the reaction product of step (c); (e) contacting the product
- 35 of step (d) with a first and a second oligonucleotide,

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the first oligonucleotide being complementary to a third sequence in the strand of the product of step (d) containing the first detectable label, the third sequence being between the polymorphic restriction site and the

5 sequence corresponding to or complementary to the first primer, the first oligonucleotide being tagged with the first member of a specific binding pair, the second oligonucleotide being complementary to a fourth sequence in the strand of the product of step (d) containing the

10 second detectable label, the fourth sequence being on the same side of the polymorphic restriction site as the third sequence, the fourth sequence not being contained within or being complementary to any of the primers, the second oligonucleotide being tagged with the first member

15 of the specific binding pair; (f) contacting the reaction product of step (e) with the second member of the specific binding pair, immobilized on a solid support; and (g) determining the ratio of the levels of the first and second detectable labels bound to the solid support,

20 a ratio of 1:0 being an indication of a homozygote containing the polymorphic restriction site, a ratio of 1:1 being an indication of a homozygote lacking the polymorphic restriction site, and a ratio of 2:1 being an indication of a heterozygote.

25 In an eighteenth aspect, the invention features a method for detecting the presence or absence of a polymorphic restriction site in a nucleic acid, involving (a) amplifying the nucleic acid by PCR using a first and second primer flanking the polymorphic restriction site,

30 the first primer containing a first sequence not complementary to or present in the nucleic acid, the second primer containing a second sequence not complementary to or present in the nucleic acid; (b) amplifying the product of step (a) by PCR using a third

35 and a fourth primer, the third primer containing the

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first sequence or a sequence complementary to the first sequence, the third primer being tagged with a first detectable label, the fourth primer containing the second sequence or a sequence complementary to the second

5 sequence, the fourth primer being tagged with a second detectable label; (c) digesting the reaction product of step (b) with the restriction endonuclease corresponding to the polymorphic restriction site; (d) denaturing the reaction product of step (c); (e) contacting the product

10 of step (d) with a first and a second oligonucleotide, the first oligonucleotide being complementary to a third sequence in the strand of the product of step (d) containing the first detectable label, the third sequence being between the polymorphic restriction site and the

15 sequence corresponding to or complementary to the second primer, the first oligonucleotide being tagged with the first member of a specific binding pair, the second oligonucleotide being complementary to a fourth sequence in the strand of the product of step (d) containing the

20 second detectable label, the fourth sequence being on the same side of the polymorphic restriction site as the third sequence, the fourth sequence not being contained within or being complementary to any of the primers, the second oligonucleotide being tagged with the first member

25 of the specific binding pair; (f) contacting the reaction product of step (e) with the second member of the specific binding pair, immobilized on a solid support; and (g) determining the ratio of the levels of the first and second detectable labels bound to the solid support,

30 a ratio of 0:1 being an indication of a homozygote containing the polymorphic restriction site, a ratio of 1:1 being an indication of a homozygote lacking the polymorphic restriction site, and a ratio of 1:2 being an indication of a heterozygote.

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In a nineteenth aspect, the invention features a method for detecting the presence or absence of a polymorphic restriction site in a nucleic acid, involving the steps of (a) amplifying the nucleic acid by PCR using  
5 a first and a second primer flanking the polymorphic restriction site, the first primer containing a first sequence not complementary to or present in the nucleic acid, the second primer containing a second sequence not complementary to or present in the nucleic acid; (b)  
10 amplifying the product of step (a) by PCR using a third and a fourth primer, the third primer containing the first sequence or a sequence complementary to the first sequence, the third primer being tagged with the first member of a first specific binding pair, the fourth  
15 primer containing the second sequence or a sequence complementary to the second sequence, the fourth primer being tagged with a detectable label; (c) digesting the reaction product of step (b) with the restriction endonuclease corresponding to the polymorphic restriction  
20 site; (d) contacting the reaction product of step (c) with the second member of the first specific binding pair, immobilized on a first solid support; (e) denaturing the reaction product of step (d) not bound to the first solid support; (f) contacting the product of  
25 step (e) with an oligonucleotide complementary to a third sequence in the strand of the product of step (e) containing the detectable label, the third sequence being between the polymorphic restriction site and the sequence corresponding to or complementary to the second primer,  
30 the oligonucleotide being tagged with the first member of a second specific binding pair; (g) contacting the reaction product of step (f) with the second member of the second specific binding pair, immobilized on a second solid support; and (h) determining the ratio of the level  
35 of the detectable label bound to the first solid support

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to the level of the detectable label bound to the second solid support, a ratio of 0:1 being an indication of a homozygote containing the polymorphic restriction site, in a case where the total amount of the reaction product from step (d) not bound to the first solid support was used in steps (e), (f), and (g); a ratio of 1:0 being an indication of a homozygote lacking the polymorphic restriction site, in a case where the total amount of the reaction product from step (d) not bound to the first solid support was used in steps (e), (f), and (g); and a ratio of 1:1 being an indication of a heterozygote, in a case where the total amount of the reaction product from step (d) not bound to the first solid support was used in steps (e), (f), and (g).

15 In a twentieth aspect the invention features a method for detecting the presence or absence of a polymorphic restriction site in a nucleic acid, involving the steps of: (a) amplifying the nucleic acid by PCR using a first and a second primer flanking the polymorphic restriction site, the first primer containing a first sequence not complementary to or present in the nucleic acid; (b) amplifying the product of step (a) by PCR using a third primer and the second primer, the third primer containing the first sequence, the third primer being tagged with a detectable label; (c) digesting the reaction product of step (b) with the restriction endonuclease corresponding to the polymorphic restriction site; (d) annealing and ligating to the single-stranded ends generated in the reaction of step (c) a first oligonucleotide tagged with the first member of a first specific binding pair; (e) contacting the reaction product of step (d) with the second member of the first specific binding pair, immobilized on a first solid support; (f) denaturing the reaction product of step (e) not bound to the first solid support; (g) contacting the

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product of step (f) with a second oligonucleotide complementary to a second sequence in the strand of the product of step (f) containing the detectable label, the second sequence being between the polymorphic restriction site and either the sequence corresponding to or complementary to the second primer or the sequence corresponding to or complementary to the first primer, the second oligonucleotide being tagged with the first member of a second specific binding pair; (h) contacting the reaction product of step (g) with the second member of the second specific binding pair, immobilized on a second solid support; and (i) determining the ratio of the level of the detectable label bound to the first solid support to the level of the detectable label bound to the second solid support, a ratio of 1:0 being an indication of a homozygote containing the polymorphic restriction site, in a case where the total amount of the reaction product from step (e) not bound to the first solid support was used in steps (f), (g), and (h); a ratio of 0:1 being an indication of a homozygote lacking the polymorphic restriction site, in a case where the total amount of the reaction product from step (e) not bound to the first solid support was used in steps (f), (g), and (h); and a ratio of 1:1 being an indication of a heterozygote; in a case where the total amount of the reaction product from step (e) not bound to the first solid support was used in steps (f), (g), and (h).

In a twenty-first aspect, the invention features a method for detecting the presence or absence of a polymorphic restriction site in a nucleic acid, involving the steps of: (a) amplifying the nucleic acid by PCR using a first and a second primer flanking the polymorphic restriction site, the first primer containing a first sequence not complementary to or present in the nucleic acid, the second primer containing a second

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sequence not complementary to or present in the nucleic acid; (b) amplifying the product of step (a) by PCR using a third and a fourth primer, the third primer containing the first sequence or a sequence complementary to the

5 first sequence, the third primer being tagged with the first member of a first specific binding pair, the fourth primer containing the second sequence or a sequence complementary to the second sequence, the fourth primer being tagged with a detectable label; (c) digesting the

10 reaction product of step (b) with the restriction endonuclease corresponding to the polymorphic restriction site; (d) contacting the reaction product of step (c) with the second member of the first specific binding pair, immobilized on a first solid support; (e)

15 denaturing the reaction product from step (d) not bound to the first solid support; (f) contacting the product of step (e) with an oligonucleotide complementary to a third sequence in the strand of the product of step (e) containing the detectable label, the third sequence being

20 between the polymorphic restriction site and the sequence corresponding to or complementary to the second primer, the oligonucleotide being immobilized on a second solid support; and (g) determining the ratio of the level of the detectable label bound to the first solid support to

25 the level of the detectable label bound to the second solid support, a ratio of 0:1 being an indication of a homozygote containing the polymorphic restriction site, in a case where the total amount of the reaction product from step (d) not bound to the first solid support was

30 used in steps (e) and (f); a ratio of 1:0 being an indication of a homozygote lacking the polymorphic restriction site, in a case where the total amount of the reaction product from step (d) not bound to the first solid support was used in steps (e) and (f); and a ratio

35 of 1:1 being an indication of a heterozygote, in a case

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where the total amount of the reaction product from step (d) not bound to the first solid support was used in steps (e) and (f).

In a twenty-second aspect, the invention features  
5 a method for detecting the presence or absence of a polymorphic restriction site in a nucleic acid, the method involving the steps of: (a) amplifying the nucleic acid by PCR using a first and a second primer flanking the polymorphic restriction site, the first primer  
10 containing a first sequence not complementary to or present in the nucleic acid; (b) amplifying the product of step (a) by PCR using a third primer and the second primer, the third primer containing the first sequence, the third primer being tagged with a detectable label;  
15 (c) digesting the reaction product of step (b) with the restriction endonuclease corresponding to the polymorphic restriction site; (d) annealing and ligating to the single-stranded ends generated in the reaction of step (c) a first oligonucleotide tagged with the first member  
20 of a first specific binding pair; (e) contacting the reaction product of step (d) with the second member of the first specific binding pair, immobilized on a first solid support; (f) denaturing the reaction product of step (e) not bound to the first solid support; (g)  
25 contacting the product of step (f) with a second oligonucleotide complementary to a second sequence in the strand of the product of step (f) containing the detectable label, the second sequence being between the polymorphic restriction site and either the sequence  
30 corresponding to or complementary to the second primer or the sequence corresponding to or complementary to the first primer, the second oligonucleotide being immobilized on a second solid support; and (h) determining the ratio of the level of the detectable  
35 label bound to the first solid support to the level of



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the detectable label bound to the second solid support, a ratio of 1:0 being an indication of a homozygote containing the polymorphic restriction site, in a case where the total amount of the reaction product from step 5 (e) not bound to the first solid support was used in steps (f) and (g); a ratio of 0:1 being an indication of a homozygote lacking the polymorphic restriction site, in a case where the total amount of the reaction product from step (e) not bound to the first solid support was 10 used in steps (f) and (g); and a ratio of 1:1 being an indication of a heterozygote, in a case where the total amount of the reaction product from step (e) not bound to the first solid support was used in steps (f) and (g).

In a twenty-third aspect, the invention features a 15 kit for detecting the presence or absence of a polymorphic restriction site in a nucleic acid, the kit containing one or more sets of a first and a second primer flanking the polymorphic restriction site, the first primer being tagged with the first member of a 20 specific binding pair, the second primer being tagged with a detectable label. In a preferred embodiment, the kit further contains the second member of the specific binding pair, immobilized on a solid support. In another preferred embodiment, the kit further contains an 25 oligonucleotide complementary to the single-stranded ends generated in the nucleic acid upon digestion of the nucleic acid with the restriction enzyme corresponding to the polymorphic restriction site, the oligonucleotide being tagged with a second detectable label.

30 In a twenty-fourth aspect, the invention features a kit for detecting the presence or absence of a polymorphic restriction site in a nucleic acid, the kit containing: (a) a first and a second primer flanking the polymorphic restriction site, the first primer being 35 tagged with a detectable label, the second primer being

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unlabeled; (b) an oligonucleotide complementary to a sequence in the strand of the nucleic acid complementary to the second primer, the sequence being between the polymorphic restriction site and the sequence  
5 complementary to the second primer, the oligonucleotide being tagged with a first member of a specific binding pair; and (c) the second member of the specific binding pair, immobilized on a solid support.

In a twenty-fifth aspect, the invention features a  
10 kit for detecting the presence or absence of a polymorphic restriction site in a nucleic acid, the kit containing: (a) a first and a second primer flanking the polymorphic restriction site, the first primer being tagged with a first detectable label, the second primer  
15 being tagged with a second detectable label; (b) a first oligonucleotide, the first oligonucleotide being complementary to a first sequence in the strand of the nucleic acid complementary to the second primer, the first sequence being between the polymorphic restriction  
20 site and either the sequence corresponding to the first primer or the sequence complementary to the second primer, the first oligonucleotide being tagged with the first member of a first specific binding pair; (c) a second oligonucleotide, the second oligonucleotide being  
25 complementary to a second sequence in the strand of the nucleic acid complementary to the first primer, the second sequence being on the same side of the polymorphic restriction site as the first sequence, the second sequence not being contained within or being  
30 complementary to either of the first or second primers, the second oligonucleotide being tagged with the first member of a second specific binding pair; (d) the second member of the first specific binding pair, immobilized on a first solid support; and (e) the second member of the  
35 second specific binding pair, immobilized on a second

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solid support. In a preferred embodiment, the first and the second specific binding pairs are identical, and the first and the second solid supports are identical.

In a twenty-sixth aspect, the invention features a  
5 kit for detecting the presence or absence of a  
polymorphic restriction site in a nucleic acid, the kit  
containing: (a) a first and a second primer flanking  
the polymorphic restriction site, the first primer being  
tagged with the first member of a first specific binding  
10 pair, the second primer being tagged with a detectable  
label; (b) the second member of the first specific  
binding pair, immobilized on a first solid support; (c)  
an oligonucleotide complementary to a first sequence in  
the strand of the nucleic acid containing the sequence  
15 corresponding to the second primer, the first sequence  
being between the polymorphic restriction site and the  
sequence corresponding to the second primer, the  
oligonucleotide being tagged with the first member of a  
second specific binding pair; and (d) the second member  
20 of the second specific binding pair, immobilized on a  
second solid support.

In a twenty-seventh aspect, the invention features  
a kit for detecting the presence or absence of a  
polymorphic restriction site in a nucleic acid, the kit  
25 containing: (a) a first and a second primer flanking  
the polymorphic restriction site, the first primer being  
tagged with a detectable label, the second primer being  
unlabeled; (b) a first oligonucleotide complementary to  
the single-stranded ends generated in the nucleic acid  
30 upon digestion of the nucleic acid with the restriction  
enzyme corresponding to the polymorphic restriction site,  
the oligonucleotide being tagged with the first member of  
a first specific binding pair; (c) the second member of  
the first specific binding pair, immobilized on a first  
35 solid support; (d) a second oligonucleotide complementary

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to a sequence in the strand of the nucleic acid complementary to the second primer, the sequence being between the polymorphic restriction site and either the sequence corresponding to the first primer or the sequence complementary to the second primer, the second oligonucleotide being tagged with the first member of a second specific binding pair; and (e) the second member of the second specific binding pair, immobilized on a second solid support.

10 In a twenty-eighth aspect, the invention features a kit for detecting the presence or absence of a polymorphic restriction site in a nucleic acid, the kit containing: (a) a first and a second primer flanking the polymorphic restriction site, the first primer being  
15 tagged with the first member of a first specific binding pair, the second primer being tagged with a detectable label; (b) the second member of the first specific binding pair, immobilized on a first solid support; and (c) an oligonucleotide complementary to a first sequence  
20 in the strand of the nucleic acid containing the sequence corresponding to the second primer, the first sequence being between the polymorphic restriction site and the sequence corresponding to the second primer, the oligonucleotide being immobilized on a second solid  
25 support.

In a twenty-ninth aspect, the invention features a kit for detecting the presence or absence of a polymorphic restriction site in a nucleic acid, the kit containing: (a) a first and a second primer flanking  
30 the polymorphic restriction site, the first primer being tagged with a detectable label, the second primer being unlabeled; (b) a first oligonucleotide complementary to the single-stranded ends generated in the nucleic acid upon digestion of the nucleic acid with the restriction  
35 enzyme corresponding to the polymorphic restriction site,

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the oligonucleotide being tagged with the first member of a first specific binding pair; (c) the second member of the first specific binding pair, immobilized on a first solid support; and (d) a second oligonucleotide  
5 complementary to a sequence in the strand of the nucleic acid complementary to the second primer, the sequence being between the polymorphic restriction site and either the sequence corresponding to the first primer or the sequence complementary to the second primer, the second  
10 oligonucleotide being immobilized on a second solid support.

In a thirtieth aspect, the invention features a kit for detecting the presence or absence of a polymorphic restriction site in a nucleic acid, the kit  
15 containing: (a) a first and a second primer flanking the polymorphic restriction site, the first primer containing a first sequence not complementary to or present in the nucleic acid, the second primer containing a second sequence not complementary to or present in the  
20 nucleic acid; (b) a third and a fourth primer, the third primer containing the first sequence or a sequence complementary to the first sequence, the third primer being tagged with the first member of a specific binding pair, the fourth primer containing the second sequence or  
25 a sequence complementary to the second sequence, the fourth primer being tagged with a detectable label. In a preferred embodiment, the kit further contains the second member of the specific binding pair, immobilized on a solid support. In another preferred embodiment, the kit  
30 further contains an oligonucleotide complementary to the single-stranded ends generated in the nucleic acid upon digestion of the nucleic acid with the restriction enzyme corresponding to the polymorphic restriction site, the oligonucleotide being tagged with a second detectable  
35 label. In a thirty-first aspect, the invention

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features a kit for detecting the presence or absence of a polymorphic restriction site in a nucleic acid, the kit containing: (a) a first and a second primer flanking the polymorphic restriction site, the first primer  
5 containing a first sequence not complementary to or present in the nucleic acid; (b) a third primer containing the first sequence, the third primer being tagged with a detectable label; (c) an oligonucleotide complementary to a second sequence in the strand of the  
10 nucleic acid containing the sequence complementary to the second primer, the second sequence being between the polymorphic restriction site and the sequence complementary to the second primer, the oligonucleotide being tagged with a first member of a specific binding  
15 pair; and (d) the second member of the specific binding pair, immobilized on a solid support.

In a thirty-second aspect, the invention features a kit for detecting the presence or absence of a polymorphic restriction site in a nucleic acid, the kit  
20 containing: (a) a first and a second primer flanking the polymorphic restriction site, the first primer containing a first sequence not complementary to or present in the nucleic acid, the second primer containing a second sequence not complementary to or present in the nucleic  
25 acid; (b) a third and a fourth primer, the third primer containing the first sequence or a sequence complementary to the first sequence, the third primer being tagged with a first detectable label, the fourth primer containing the second sequence or a sequence complementary to the  
30 second sequence, the fourth primer being tagged with a second detectable label; (c) a first oligonucleotide, the first oligonucleotide being complementary to a third sequence in the strand of the nucleic acid complementary to the second primer, the third sequence being between  
35 the polymorphic restriction site and either the sequence

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complementary to the second primer or the sequence corresponding to the first primer, the first oligonucleotide being tagged with the first member of a first specific binding pair, (d) a second

5 oligonucleotide, the second oligonucleotide being complementary to a fourth sequence in the strand of the nucleic acid complementary to the first primer, the fourth sequence being on the same side of the polymorphic restriction site as the third sequence, the fourth

10 sequence not being contained within or being complementary to any of the primers, the second oligonucleotide being tagged with the first member of a second specific binding pair; (e) the second member of the first specific binding pair, immobilized on a first

15 solid support; and (f) the second member of the second specific binding pair, immobilized on a second solid support. In a preferred embodiment, the first and the second specific binding pairs are identical, and the first and the second solid supports are identical.

20 In a thirty-third aspect, the invention features a kit for detecting the presence or absence of a polymorphic restriction site in a nucleic acid, the kit containing: (a) a first and a second primer flanking the polymorphic restriction site, the first primer

25 containing a first sequence not complementary to or present in the nucleic acid, the second primer containing a second sequence not complementary to or present in the nucleic acid; (b) a third and a fourth primer, the third primer containing the first sequence or a sequence

30 complementary to the first sequence, the third primer being tagged with the first member of a first specific binding pair, the fourth primer containing the second sequence or a sequence complementary to the second sequence, the fourth primer being tagged with a

35 detectable label; (c) the second member of the first

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specific binding pair, immobilized on a first solid support; (d) an oligonucleotide complementary to a third sequence in the strand of the nucleic acid corresponding to the second primer, the sequence being between the  
5 polymorphic restriction site and the sequence corresponding to the second primer, the oligonucleotide being tagged with the first member of a second specific binding pair; and (e) the second member of the second specific binding pair, immobilized on a second solid  
10 support.

In a thirty-fourth aspect, the invention features a kit for detecting the presence or absence of a polymorphic restriction site in a nucleic acid, the kit containing: (a) a first and a second primer flanking  
15 the polymorphic restriction site, the first primer containing a first sequence not complementary to or present in the nucleic acid; (b) a third primer containing the first sequence, the third primer being tagged with a detectable label; (c) a first  
20 oligonucleotide complementary to the single-stranded ends generated in the nucleic acid upon digestion of the nucleic acid with the restriction enzyme corresponding to the polymorphic restriction site, the oligonucleotide being tagged with the first member of a first specific  
25 binding pair; (d) the second member of the first specific binding pair, immobilized on a first solid support; (e) a second oligonucleotide complementary to a second sequence in the strand of the nucleic acid corresponding to the first primer, the second sequence being between the  
30 polymorphic restriction site and either the sequence complementary to the second primer or the sequence corresponding to the first primer, the second oligonucleotide being tagged with the first member of a second specific binding pair; and (f) the second member



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of the second specific binding pair, immobilized on a second solid support.

In a thirty-fifth aspect, the invention features a kit for detecting the presence or absence of a polymorphic restriction site in a nucleic acid, the kit containing: (a) a first and a second primer flanking the polymorphic restriction site, the first primer containing a first sequence not complementary to or present in the nucleic acid, the second primer containing a second sequence not complementary to or present in the nucleic acid; (b) a third and a fourth primer, the third primer containing the first sequence or a sequence complementary to the first sequence, the third primer being tagged with the first member of a first specific binding pair, the fourth primer containing the second sequence or a sequence complementary to the second sequence, the fourth primer being tagged with a detectable label; (c) the second member of the first specific binding pair, immobilized on a first solid support; and (d) an oligonucleotide complementary to a third sequence in the strand of the nucleic acid corresponding to the second primer, the third sequence being between the polymorphic restriction site and the sequence corresponding to the second primer, the oligonucleotide being immobilized on a second solid support.

In a thirty-sixth aspect, the invention features a kit for detecting the presence or absence of a polymorphic restriction site in a nucleic acid, the kit containing: (a) a first and a second primer flanking the polymorphic restriction site, the first primer containing a first sequence not complementary to or present in the nucleic acid; (b) a third primer containing the first sequence, the third primer being tagged with a detectable label; (c) a first

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oligonucleotide complementary to the single-stranded ends generated in the nucleic acid upon digestion of the nucleic acid with the restriction enzyme corresponding to the polymorphic restriction site, the oligonucleotide  
5 being tagged with the first member of a first specific binding pair; (d) the second member of the first specific binding pair, immobilized on a first solid support; and (e) a second oligonucleotide complementary to a second sequence in the strand of the nucleic acid corresponding  
10 to the first primer, the sequence being between the polymorphic restriction site and either the sequence corresponding to or complementary to the second primer or the sequence corresponding to or complementary to the first primer, the second oligonucleotide being  
15 immobilized on a second solid support.

In a thirty-seventh aspect, the invention features a method for detecting the presence or absence of a polymorphic restriction site in a nucleic acid, involving the steps of: (a) amplifying the nucleic acid by PCR  
20 using a first and a second primer flanking the polymorphic restriction site, whereby the resultant PCR product is of a defined size readily resolved by gel electrophoresis; (b) digesting the PCR product of step (a) with the restriction endonuclease corresponding to  
25 the polymorphic restriction site, the digestion products being differentially sized; (c) separating the reaction products of step (b) by gel electrophoresis; and (d) detecting the separated reaction products, the presence of only uncleaved products being an indication  
30 of a homozygote lacking the polymorphic restriction site, the presence of only cleaved products being an indication of a homozygote containing the polymorphic restriction site, and the presence of both cleaved and uncleaved products being an indication of a heterozygote. In a  
35 preferred embodiment, one or both of the first and second

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primers are tagged with a detectable label. In another preferred embodiment, the PCR product is 100-1000 base pairs in length.

In a thirty-eighth aspect, the invention features  
5 a kit for detecting the presence or absence of a  
polymorphic restriction site in a nucleic acid, the kit  
containing: a first and a second primer flanking the  
polymorphic restriction site and capable of generating a  
PCR product of a defined size that is readily resolved by  
10 gel electrophoresis. In a preferred embodiment, the  
first and/or the second primers are detectably labeled.  
In another preferred embodiment, the PCR product  
generated is between 100 and 1000 base pairs in length.

In a thirty-ninth aspect, the invention features a  
15 method for identifying a polymorphic restriction site in  
a nucleic acid, involving the steps of: (a) digesting DNA  
isolated from a first sample with a first restriction  
endonuclease; (b) ligating to each of the ends of the  
reaction products of step (a) a first adaptor; (c)  
20 digesting the products of step (b) with a second  
restriction endonuclease; (d) ligating to each of the  
ends of the reaction products generated in step (c) a  
second adaptor; (e) amplifying the reaction products of  
step (d) by PCR using a first primer complementary to the  
25 first adaptor and a second primer complementary to the  
second adaptor, the second primer being tagged with a  
first member of a specific binding pair (preferably,  
biotin); (f) in a separate set of reactions, digesting  
DNA isolated from a second sample with the first  
30 restriction endonuclease; (g) ligating to each of the  
ends of the reaction products of step (f) a third  
adaptor; (h) digesting the products of step (g) with the  
second restriction endonuclease; (i) denaturing the  
products of step (e) and the products of step (h); (j)  
35 combining the denatured products of step (i) under

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conditions allowing hybridization; (k) contacting the hybridization products of step (j) with the second member of the specific binding pair (preferably, avidin), the second member being immobilized on a solid support; (l) recovering the hybridization products captured on the solid support; and (m) amplifying the products obtained in step (l) by PCR using a primer complementary to the third adaptor, an amplified product being an indication of a polymorphic restriction site corresponding to the second restriction endonuclease.

In a related aspect, the invention features a kit for identifying a polymorphic restriction site in a nucleic acid, the kit comprising: (a) a first DNA adaptor, a second DNA adaptor, and a third DNA adaptor, the first and third DNA adaptors having regions complementary to the ends generated by a first restriction endonuclease ends but differing in overall sequence and the second DNA adaptor having a region complementary to the ends generated by a second restriction endonuclease, the second restriction endonuclease site corresponding to the polymorphic restriction site; and (b) a first primer, a second primer, and a third primer, the first primer being complementary to the first DNA adaptor, the second primer being complementary to the second DNA adaptor and being tagged with a first member of a specific binding pair, and the third primer being complementary to the third DNA adaptor. This kit may further comprises the second member of the specific binding pair immobilized on a solid support.

In a preferred embodiment of various of the above aspects, multiple polymorphic restriction sites are detected by the method or kit. In preferred embodiments of various of the above aspects, the detectable label is selected from the group consisting of digoxigenin,

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fluorescent labels (e.g., fluorescein and rhodamine), enzymes (e.g., horseradish peroxidase and alkaline phosphatase), biotin (which can be detected by anti-biotin specific antibodies or enzyme-conjugated avidin derivatives), radioactive labels (e.g.,  $^{32}\text{P}$  and  $^{125}\text{I}$ ), colorimetric reagents, and chemiluminescent reagents.

In other preferred embodiments of various of the above aspects, the specific binding pairs are selected from the group consisting of avidin-biotin, streptavidin-biotin, hybridizing nucleic acid pairs, interacting protein pairs, antibody-antigen pairs, reagents containing chemically reactive groups (e.g., reactive amino groups), and nucleic acid sequence-nucleic acid binding protein pairs. In related preferred embodiments of various of the above aspects, the solid supports used in the methods of the invention are selected from the group consisting of agarose, acrylamide, or polystyrene beads; polystyrene microtiter plates (for use in, e.g., ELISA); and nylon and nitrocellulose membranes (for use in, e.g., dot or slot blot assays).

The term "heterozygote," as used herein, refers to an individual with different alleles at corresponding loci on homologous chromosomes. Accordingly, the term "heterozygous," as used herein, describes an individual or strain having different allelic genes at one or more paired loci on homologous chromosomes.

The term "homozygote," as used herein, refers to an individual with the same allele at corresponding loci on homologous chromosomes. Accordingly, the term "homozygous," as used herein, describes an individual or a strain having identical allelic genes at one or more paired loci on homologous chromosomes.

The term "corresponding" as used herein to describe a nucleic acid strand, e.g., a nucleic acid strand corresponding to a particular PCR primer, is meant

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to indicate that the strand contains the sequence of the particular PCR primer. When used to compare a polymorphic restriction site to a restriction endonuclease site, the term again indicates that the two  
5 sequences are identical.

An advantage of certain detection methods of the present invention over many other methods used to detect genetic polymorphisms is that gel electrophoresis is not required in the analysis. Thus, the methods of the  
10 present invention are readily adaptable for automation, allowing large numbers of samples to be processed in relatively short periods of time, at lower costs. In addition, in several variations of the methods of the invention (see, e.g., Examples III and IV below),  
15 internal controls are provided, thus controlling for variability detected by different practitioners. Furthermore, in several of the variations of the methods of the invention (see Examples III - VIII below), an oligonucleotide probe hybridizing to a sequence in the  
20 PCR product internal to the primers is used to purify the products, thus allowing a reduction in background problems associated with PCR amplification.

Those detection methods of the invention utilizing gel electrophoresis are also advantageous because they  
25 provide a rapid and inexpensive approach to the identification of large numbers of PCR-based genetic and RFLP markers.

The method of the invention useful for cloning genetic polymorphisms also represents an improvement over  
30 current methods. Because the process of selecting out a tagged (e.g., biotinylated) DNA having a polymorphism involves a specific hybridization step, candidate DNA from any source may be utilized. For example, DNA from random clones, cDNA libraries, YAC libraries, or any  
35 other DNA collection may be screened; pure preparations

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of genomic DNAs are not required. Moreover, like other methods of the invention, this cloning procedure is rapid and inexpensive.

All methods of the invention are useful in  
5 clinical diagnostic testing, genomic mapping, positional cloning of genes defined by mutation (such as those that cause inherited disease in humans or resistance to pathogens in crop plants), DNA fingerprinting (e.g., for forensic analysis and paternity testing), crop and  
10 livestock breeding programs, and other related applications.

In one particular example, the detection methods of the invention are useful for bacterial typing utilizing known conserved polymorphic sequences  
15 diagnostic of the bacterium. In one application, this approach is useful for distinguishing one bacterium from another (e.g., for the identification of Salmonella in a food sample); polymorphism-containing sequences preferred for this approach include those present in conserved  
20 ribosomal RNA genes. In another application, this approach is useful for screening bacteria (e.g., clinical isolates) for antibiotic resistance; in this case, known polymorphic restriction sites within the antibiotic resistance marker are utilized. The instant methods of  
25 bacterial typing decrease false positive results frequently obtained using current PCR-based techniques.

#### Detailed Description

The drawings are first described.

#### Drawings

30 Fig. 1 is a schematic of a RFLP detection method involving the use of a first PCR primer tagged with a detectable label (X) and a second PCR primer tagged with the first member of a specific binding pair (Y). After amplification by PCR, the products are digested with the

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restriction endonuclease (R) corresponding to the polymorphic restriction site; contacted with the second member of the specific binding pair immobilized on a solid support, and the level of the detectable label (X) bound to the solid support is determined.

Fig. 2 is a schematic of a RFLP detection method involving the use of a first PCR primer tagged with a first detectable label (X) and a second PCR primer tagged with the first member of a specific binding pair (Y). After amplification by PCR, the products are digested with the restriction endonuclease (R) corresponding to the polymorphic restriction site, and an oligonucleotide tagged with a second detectable label (Z) is annealed and ligated to the single-stranded ends generated in the digestion. The reaction is then contacted with the second member of the specific binding pair bound to a solid support, and the levels of the first and second detectable labels (X and Z) bound to the solid support are determined.

Fig. 3 is a schematic of a RFLP detection method involving the use of a first PCR primer tagged with a detectable label (P1) and a second unlabeled PCR primer (P2). After amplification by PCR, half of the reaction (or one of the identical reactions if carried out in duplicate) is digested with the restriction endonuclease (R) corresponding to the polymorphic restriction site. Both digested and undigested reactions are then denatured and contacted with an oligonucleotide tagged with the first member of a specific binding pair, the oligonucleotide being complementary to the P1 strand and located to the right of the restriction site (R) near to, but not overlapping, primer P2. The reactions are then contacted with the second member of the specific binding pair immobilized on a solid support, and the levels of P1 in digested versus undigested reactions are compared.



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Fig. 4 is a schematic of a RFLP detection method involving the use of a first PCR primer tagged with a first detectable label (P1) and a second PCR primer tagged with a second detectable label (P2). After  
5 amplification by PCR, the products are digested with the restriction endonuclease (R) corresponding to the polymorphic restriction site, denatured, and contacted with a first oligonucleotide complementary to the P1 strand and located to the right of the restriction site  
10 (R) near to, but not overlapping primer P2, and a second oligonucleotide complementary to the P2 strand and located to the right of the restriction site (R) near to, but not overlapping the sequence complementary to primer P2. Both the first and second oligonucleotides are  
15 tagged with the first member of a specific binding pair (Y). The reactions are then contacted with the second member of the specific binding pair immobilized on a solid support, and the ratio of P1 to P2 bound to the solid support is determined.

20 Fig. 5 is a schematic of a RFLP detection method involving the use of a first PCR primer tagged with a detectable label (X) and a second PCR primer tagged with the first member of a first specific binding pair (Y). After amplification by PCR, the products are digested  
25 with the restriction enzyme (R) corresponding to the polymorphic restriction site, and are contacted with the second member of the first specific binding pair immobilized on a first solid support. The filtrate is then bound to a solid support with the anchor sequence  
30 (or contacted with an oligonucleotide complementary to the X strand between the restriction site (R) and the label (X), the oligonucleotide being tagged with the first member of a second specific binding pair, and then contacted with the second member of the second specific  
35 binding pair immobilized on a second solid support), and

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the levels of the detectable label bound to the first solid support and the anchor sequence (or second solid support) are determined.

Fig. 6 is a schematic of a RFLP detection method involving the use of a first unlabeled PCR primer and a second PCR primer tagged with a detectable label (X). After amplification by PCR, the products are digested with the restriction enzyme (R) corresponding to the polymorphic restriction site, and contacted with an oligonucleotide complementary to the single-stranded ends generated in the digestion, the oligonucleotide being tagged with the first member of a specific binding pair. The products are then contacted with the second member of the first specific binding pair, bound to a first solid support. The filtrate is then bound to a solid support with the anchor sequence (or contacted with an oligonucleotide complementary to the X strand, the oligonucleotide being tagged with the first member of a second specific binding pair, and then contacted with the second member of the second specific binding pair immobilized on a second solid support), and the levels of the detectable label bound to the first solid support and the anchor sequence (or second solid support) are determined.

Fig. 7 is a schematic of a RFLP detection method involving the use of PCR primers flanking the polymorphic restriction site (the "Alu I" site). Following PCR amplification, the reaction products are digested with the restriction endonuclease corresponding to the polymorphic restriction site (Alu I), and the fragments are run on an agarose gel. The separated fragments are detected as an indication of the presence or absence of the polymorphic marker.

Fig. 8 is a schematic of a typical gel analysis according the method described in Fig. 7.

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Fig. 9 is a schematic of a method for cloning polymorphic restriction fragments.

Methods for generating and detecting genetic polymorphisms

- 5           The present invention provides several methods for detecting Cleaved Amplified Polymorphic Sequences (CAPS; Konieczny et al., The Plant Journal 4(2):403-410, 1993). In the CAPS method, a nucleic acid containing a polymorphic restriction site is amplified using primers  
10 flanking the restriction site. The resulting PCR product is digested with the restriction endonuclease corresponding to the polymorphic restriction site, and the digested products are analyzed by gel electrophoresis.
- 15           The detection methods of the present invention vary greatly from one another in detail, however they share three central features: (1) the nucleic acid containing the polymorphic restriction site is amplified by PCR using differently labeled primers flanking the  
20 polymorphic restriction site, (2) the resulting PCR product is digested with the restriction endonuclease corresponding to the polymorphic restriction site (which will cleave the DNA of some individuals but not cleave the DNA of others, depending on the presence of the  
25 polymorphism), and (3) the resulting digestion products are analyzed by detection of the labels they contain, and/or labels attached to oligonucleotides complementary to the digestion products, in order to determine the identity of the polymorphic restriction site. The  
30 methods of the invention allow rapid and efficient analyses of a large number of samples.

The nucleic acid sample containing the polymorphic restriction site being analyzed can be obtained from any source, e.g., a tissue homogenate, blood, amniotic fluid,

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and chorionic villus samples; and can be obtained from these sources using standard methods. Only a minute quantity of nucleic acid is required, and can be DNA or RNA (in the case of RNA, a reverse transcription step is  
5 required before the PCR step). The polymerase chain reactions (PCR) used in the methods of the present invention are carried out using standard methods (see, e.g., Ausubel et al., Current Protocols in Molecular Biology, John Wiley and Sons, New York, 1989; Erlich, PCR  
10 Technology, Stockton Press, New York, 1989; Innis et al., PCR Protocols: A Guide to Methods and Applications, Academic Press, Harcourt Brace Javanovich, New York, 1990; Sambrook et al., Molecular Cloning: A Laboratory Manual, Cold Spring Harbor Laboratory Press, Cold Spring  
15 Harbor, New York, 1989). Restriction enzyme digestion is also carried out by standard methods using any of a number of available restriction endonucleases (see, e.g., Ausubel et al., *supra*; New England Biolabs, Beverly, MA).

The primers and oligonucleotides used in the  
20 methods of the present invention are preferably DNA, and can be synthesized using standard techniques and, when appropriate, detectably labeled using standard methods (Ausubel et al., Current Protocols in Molecular Biology, John Wiley and Sons, New York, 1989). Detectable labels  
25 that can be used to tag the primers and oligonucleotides used in the methods of the invention include, but are not limited to, digoxigenin, fluorescent labels (e.g., fluorescein and rhodamine), enzymes (e.g., horseradish peroxidase and alkaline phosphatase), biotin (which can  
30 be detected by anti-biotin specific antibodies or enzyme-conjugated avidin derivatives), radioactive labels (e.g.,  $^{32}\text{P}$  and  $^{125}\text{I}$ ), colorimetric reagents, and chemiluminescent reagents. The labels used in the methods of the invention are detected using standard methods.

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The specific binding pairs useful in the methods of the invention include, but are not limited to, avidin-biotin, streptavidin-biotin, hybridizing nucleic acid pairs, interacting protein pairs, antibody-antigen pairs, 5 reagents containing chemically reactive groups (e.g., reactive amino groups), and nucleic acid sequence-nucleic acid binding protein pairs.

The solid supports useful in the methods of the invention include, but are not limited to, agarose, 10 acrylamide, or polystyrene beads; polystyrene microtiter plates (for use in, e.g., ELISA); and nylon and nitrocellulose membranes (for use in, e.g., dot or slot blot assays).

The methods of the invention can be facilitated by 15 the use of kits which contain the reagents required for carrying out the assays. The kits can contain reagents for carrying out the generation or analysis of a single polymorphic restriction site (for use in, e.g., diagnostic methods), or multiple polymorphic restriction 20 sites (for use in, e.g., genomic mapping). When multiple samples are analyzed, multiple sets of the appropriate primers and oligonucleotides are provided in the kit. In addition to the primers and oligonucleotides required for carrying out the various methods, the kits may contain 25 the enzymes used in the methods, and the reagents for detecting the labels, e.g., the substrates for enzyme labels, etc.

As discussed above, the invention provides methods and kits for generating and detecting the presence or 30 absence of a polymorphic restriction site in a nucleic acid. Examples I-IX describe eight variations of the methods of the invention. Example X describes a preferred use for the methods of the invention. Example XI describes a preferred method for cloning polymorphic 35 restriction fragments. The following examples are meant

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to illustrate, but not limit, the methods of the present invention. Other suitable modifications and adaptations of the variety of conditions and parameters of molecular biology which are obvious to those skilled in the art are within the spirit and scope of the present invention.

### EXAMPLES

#### Example I.

In this method, the nucleic acid containing the polymorphism is amplified by PCR using a first and a second primer flanking the polymorphic restriction site, the first primer being tagged with the first member of a specific binding pair, the second primer being tagged with a detectable label. The resulting PCR product is digested with the restriction endonuclease corresponding to the polymorphic restriction site and the digested products are contacted with the second member of the specific binding pair, immobilized on a solid support. The level of the detectable label bound to the solid support is then measured. The presence of the detectable label bound to the solid support is an indication of the absence of the polymorphic restriction site in the nucleic acid, while the absence of the detectable label bound to the solid support is an indication of the presence of the polymorphic restriction site in the nucleic acid. An embodiment of this method is shown in Fig. 1.

#### Example II.

This method is identical to that described in Example I, with the added step of annealing and ligating to the single-stranded ends generated in the digestion reaction, an oligonucleotide tagged with a second detectable label. After applying the reaction to the second member of the specific binding pair, the levels of

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both the first and the second detectable labels bound to the solid support are determined. The presence of only the first detectable label bound to the solid support is an indication of a homozygote lacking the polymorphic  
5 restriction site, the presence of only the second detectable label bound to the solid support is an indication of a homozygote containing the polymorphic restriction site, and the presence of both the first and the second detectable labels bound to the solid support  
10 is an indication of a heterozygote. An embodiment of this method is shown in Fig. 2.

### Example III.

In this method, the nucleic acid is amplified using a first and a second primer flanking the  
15 polymorphic restriction site, the first primer being tagged with a detectable label, the second primer being unlabeled. A portion of the PCR reaction is digested with the restriction endonuclease corresponding to the polymorphic restriction site, while another portion is  
20 left undigested. Both the digested and undigested portions are then denatured, and contacted with an oligonucleotide tagged with the first member of a specific binding pair. The oligonucleotide is complementary to a sequence in the strand of the PCR  
25 product containing the detectable label, the sequence being between the polymorphic restriction site and the sequence complementary to the second primer.

The reaction is then contacted with the second member of the specific binding pair, immobilized on a  
30 solid support, and the ratio of the levels of the detectable label bound to the solid support between undigested and digested samples is determined. A ratio of 1:0 between equivalent portions of undigested and digested samples is an indication of a homozygote

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containing the polymorphic restriction site, a ratio of 1:1 between equivalent portions of undigested and digested samples is an indication of a homozygote lacking the polymorphic restriction site, and a ratio of 2:1  
5 between equivalent portions of undigested and digested samples is an indication of a heterozygote. While the sample volumes used for detection and comparison need not be equivalent, the appropriate calculations must be carried out to account for this adjustment prior to  
10 determining the ratio of detectable label in digested and undigested samples. An embodiment of this method is shown in Fig. 3.

Example IV.

In this method, the nucleic acid is amplified by  
15 PCR using a first primer and a second primer flanking the polymorphic restriction site, the first primer being tagged with a first detectable label, and the second primer being tagged with a second detectable label.

The PCR product is digested with the restriction  
20 endonuclease corresponding to the polymorphic restriction site, denatured, and contacting with a first and a second oligonucleotide. The first oligonucleotide is complementary to a first sequence in the strand of the PCR product containing the first detectable label, the  
25 first sequence being between the polymorphic restriction site and the sequence corresponding to the first primer. The first oligonucleotide is tagged with the first member of a first specific binding pair. The second oligonucleotide is complementary to a second sequence in  
30 the strand of the PCR product containing the second detectable label. The second sequence is on the same side of the polymorphic restriction site as the first sequence, and is not contained within, or complementary to, either the first or the second primer. The second



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oligonucleotide is tagged with the first member of a second specific binding pair.

A first portion of the reaction is then contacted with the second member of the first specific binding pair, immobilized on a first solid support, while a second portion of the reaction is contacted with the second member of the second specific binding pair, immobilized on a second solid support. The ratio of the levels of the first and second detectable labels bound to the first and second solid supports is then determined. A ratio of 1:0 between equivalent amounts of the first and second portions is an indication of a homozygote containing the polymorphic restriction site, a ratio of 1:1 between equivalent amounts of the first and second portions is an indication of a homozygote lacking the polymorphic restriction site, and a ratio of 2:1 between equivalent amounts of the first and second portions is an indication of a heterozygote.

In the case where the first sequence (to which the first oligonucleotide is complementary) in the strand containing the first detectable label is between the polymorphic restriction site and the sequence complementary to the second primer, the ratios differ, as follows. The ratio of the levels of the first and second detectable labels bound to the first and second solid supports is 0:1 between equivalent amounts of the first and second portions in the case of a homozygote containing the polymorphic restriction site. The ratio is 1:1 between equivalent amounts of the first and second portions in the case of a homozygote lacking the polymorphic restriction site, and the ratio is 1:2 between equivalent amounts of the first and second portions in the case of a heterozygote. An embodiment of this method is shown in Fig. 4.

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Example V.

In this method, the nucleic acid is amplified by PCR using a first and a second primer flanking the polymorphic restriction site, the first primer being  
5 tagged with the first member of a first specific binding pair, the second primer being tagged with a detectable label. The PCR product is digested with the restriction endonuclease corresponding to the polymorphic restriction site, and the reaction is then contacted with the second  
10 member of the first specific binding pair, immobilized on a first solid support.

The material not bound to the first solid support is denatured and contacted with an oligonucleotide complementary to a sequence in the strand of the PCR  
15 product containing the detectable label. The sequence is between the polymorphic restriction site and the sequence corresponding to the second primer, and the oligonucleotide is tagged with the first member of a second specific binding pair. The reaction is then  
20 contacted with the second member of the second specific binding pair, immobilized on a second solid support, and the ratio of the level of the detectable label bound to the first solid support compared to the level of the detectable label bound to the second solid support is  
25 determined. A ratio of 0:1 is an indication of a homozygote containing the polymorphic restriction site, a ratio of 1:0 is an indication of a homozygote lacking the polymorphic restriction site, and a ratio of 1:1 is an indication of a heterozygote. These ratios are correct  
30 in cases where the total amount of the material not bound to the first solid support is used in the following steps, and should be adjusted accordingly, if a different amount of the material is used. An embodiment of this method is shown in Fig. 5.

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Example VI.

In this method, the nucleic acid is amplified by PCR using a first and a second primer flanking the polymorphic restriction site, the first primer being  
5 tagged with a detectable label, the second primer being unlabeled. The PCR product is digested with the restriction endonuclease corresponding to the polymorphic restriction site, and a first oligonucleotide tagged with the first member of a first specific binding pair is  
10 annealed and ligated to the single-stranded ends generated in the digestion reaction. The reaction is then contacted with the second member of the first specific binding pair, immobilized on a first solid support.

15 The material not bound to the first solid support is denatured, and contacted with a second oligonucleotide complementary to a sequence in the strand of the PCR product containing the detectable label, the sequence being between the polymorphic restriction site and either  
20 the sequence corresponding to the first primer or the sequence complementary to the second primer. The second oligonucleotide is tagged with the first member of a second specific binding pair. The reaction is then contacted with the second member of the second specific  
25 binding pair, immobilized on a second solid support, and the ratio of the level of the detectable label bound to the first solid support compared to the level of the detectable label bound to the second solid support is determined. A ratio of 1:0 is an indication of a  
30 homozygote containing the polymorphic restriction site, a ratio of 0:1 is an indication of a homozygote lacking the polymorphic restriction site, and a ratio of 1:1 is an indication of a heterozygote. These ratios are correct in cases where the total amount of the material not bound  
35 to the first solid support is used in the following

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steps, and should be adjusted accordingly, if a different amount of the material is used. An embodiment of this method is shown in Fig. 6.

Example VII.

5           In this method, the nucleic acid is amplified by PCR using a first and a second primer flanking the polymorphic restriction site, the first primer being tagged with the first member of a first specific binding pair, the second primer being tagged with a detectable  
10 label. The PCR product is digested with the restriction endonuclease corresponding to the polymorphic restriction site, and contacted with the second member of the first specific binding pair, immobilized on a first solid support.

15           The material not bound to the first solid support is denatured and contacted with an oligonucleotide complementary to a sequence in the strand of the PCR product containing the detectable label. The sequence is between the polymorphic restriction site and the sequence  
20 corresponding to the second primer, and the oligonucleotide is immobilized on a second solid support (e.g., a nylon or nitrocellulose membrane).

          The ratio of the level of detectable label bound to the first solid support to the level of detectable  
25 label bound to the second solid support is then determined. A ratio of 0:1 is an indication of a homozygote containing the polymorphic restriction site, a ratio of 1:0 is an indication of a homozygote lacking the polymorphic restriction site, and a ratio of 1:1 is an  
30 indication of a heterozygote. These ratios are correct in cases where the total amount of the material not bound to the first solid support is used in the following steps, and should be adjusted accordingly, if a different

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amount of the material is used. An embodiment of this method is shown in Fig. 5.

Example VIII.

In this method, the nucleic acid is amplified by  
5 PCR using a first and a second primer flanking the  
polymorphic restriction site, the first primer being  
tagged with a detectable label, the second primer being  
unlabeled. The PCR product is digested with the  
restriction endonuclease corresponding to the polymorphic  
10 restriction site, and a first oligonucleotide tagged with  
the first member of a first specific binding pair is  
annealed and ligated to the single-stranded ends  
generated in the digestion reaction. The reaction is  
contacted with the second member of the first specific  
15 binding pair, immobilized on a first solid support. The  
material not bound to the first solid support is  
denatured, and contacted with a second oligonucleotide  
complementary to a sequence in the strand of the PCR  
product containing the detectable label. The sequence is  
20 between the polymorphic restriction site and either the  
sequence corresponding to the first primer or the  
sequence complementary to the second primer, and the  
second oligonucleotide is immobilized on a second solid  
support (e.g., a nylon or nitrocellulose membrane).  
25 The ratio of the level of the detectable label  
bound to the first solid support to the level of the  
detectable label bound to the second solid support is  
then determined. A ratio of 1:0 is an indication of a  
homozygote containing the polymorphic restriction site, a  
30 ratio of 0:1 is an indication of a homozygote lacking the  
polymorphic restriction site, and a ratio of 1:1 is an  
indication of a heterozygote. These ratios are correct  
in cases where the total amount of the material not bound  
to the first solid support is used in the following

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steps, and should be adjusted accordingly, if a different amount of the material is used. An embodiment of this method is shown in Fig. 6.

PCR primers containing nucleic acid tags on their 5' ends can also be used in the methods of the invention. These primers can be used in pairs, or in combination with un-tagged primers, in the initial cycles of PCR, followed by the addition of a "universal primer(s)" complementary to the nucleic acid tags in the first primers, and contain detectable labels (e.g., biotin, fluorescent, or ELISA tags). The use of nucleic acid tagged primers in the early rounds of PCR is a cost-effective measure, as only one set of primers, the universal primers, which can be used in the analysis of many different polymorphic sites, need to be detectably labeled. The sets of primers specific for individual polymorphic restriction sites do not have to be tagged with detectable labels, but rather need only to be complementary to the universal primers in their 5' ends.

20 Example IX.

In another method of the invention, the nucleic acid is amplified by PCR using a first and a second primer flanking the polymorphic restriction site. The PCR product is digested with the restriction endonuclease corresponding to the polymorphic restriction site, and, as shown in Fig. 7, the digestion products are run on a gel (preferably an agarose gel). To simplify the gel reading, the first and second primers are preferably designed to generate a PCR product that is easily resolvable on an agarose gel (that is, preferably larger than 100 base pairs and smaller than 1000 base pairs), and the polymorphic restriction site is preferably

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located at an asymmetric position within the amplified fragment. Using this technique, short gel runs can be used for analysis, and the cleaved products easily detected. In the particular example shown in Fig. 8, 5 primers are designed to produce PCR amplified products of 300 base pairs, and cleavage at the RFLP site yields products of 200 base pairs and 100 base pairs.

In a preferred method of carrying out this method, sets of primer pairs are provided which detect a number 10 of RFLP markers. Each set of primers, for example, may be provided in one of the wells of a 96-well microtiter plate, and PCR reactions run independently. Following restriction digestion, the reaction products are transferred to an agarose gel and separated by 15 electrophoresis. A typical result of this method is shown in Fig. 8.

Detection of the amplified and cleaved products after electrophoretic separation may be carried out by standard methods of DNA staining (e.g., ethidium bromide 20 staining) or blotting (e.g., Southern blotting). Alternatively, one or both of the PCR primers may be detectably labeled, and the labels detected as described above.

#### Example X.

25 A preferred use of the methods of the invention is in conjunction with a method called RFLP subtraction. RFLP subtraction provides a large number of polymorphic genetic markers, while the methods of the present invention provide efficient methods for their analysis.

30 Carrying out RFLP subtraction results in the purification of fragments that are present in one population (the tracer) but absent in another (the driver). Purification is achieved by removing all of the fragments in the tracer DNA that have counterparts in the

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driver DNA using subtractive hybridization (Innis et al., PCR Protocols: A Guide to Methods and Applications, Academic Press, Harcourt Brace Javanovich, New York, 1990). In RFLP subtraction, the tracer is a size  
5 fraction of digested DNA from one strain and the driver is the same size fraction from a polymorphic strain. The products obtained after removing the common sequences are RFLPs; they are sized tracer fragments whose driver counterparts are not found in the same size fraction.

10        There are three steps in RFLP subtraction: preparation of the driver and tracer, subtractive hybridization, and removal of non-hybridizing sequences from the tracer. To prepare the driver and tracer DNA, genomic DNA from two different strains is digested with a  
15 restriction endonuclease, and the ends of the restriction fragments from each strain are capped with different oligonucleotide adapters. The low molecular weight fragments are then purified from a slice of an agarose gel and amplified using one of the adapter strands as a  
20 PCR primer. A biotinylated primer can be used to amplify the driver so that driver DNA can be removed following the subtractive hybridizations by binding to avidin coated beads.

      Three rounds of subtractive hybridization are  
25 performed to remove tracer sequences that also occur in the driver. A small amount of tracer is mixed with an excess of biotinylated driver, the mixture is denatured and allowed to re-anneal. Most tracer sequences will hybridize to complementary biotinylated driver strands.  
30 Some tracer sequences, however, are not represented in the driver because they reside on large restriction fragments (i.e., they are RFLPs) or are missing from the driver genome. These fragments will have no complementary biotinylated strands with which to anneal.  
35 The biotinylated driver DNA, and any tracer that has



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annealed to it, is then removed using avidin-coated beads. The unbound fraction is then subjected to two more rounds of subtractive hybridization, tracer DNA remaining after the third round is amplified, and poorly  
5 hybridizing sequences are removed.

Example XI.

Figure 9 shows a preferred method for cloning polymorphic restriction fragments. The object of this method is to clone restriction fragments from organism B  
10 (generated by restriction endonuclease A) that do not contain cleavage sites for restriction endonuclease B, and which correspond to restriction fragments in organism A (generated by restriction endonuclease A) that do contain at least one restriction site for restriction  
15 endonuclease B. These polymorphic restriction fragments are useful as CAPS markers for the detection methods described above.

Referring to the method outlined in Fig. 9, in step A, genomic DNA isolated from polymorphic individuals  
20 A and B is separately digested with restriction enzyme A, which preferably leaves so-called sticky ends. An oligonucleotide adaptor (#1), with complementary sticky ends, is ligated to the restriction fragments from individual A. A different oligonucleotide adaptor (#3)  
25 is ligated to the restriction fragments from individual B.

In step B, the restriction fragments from step A are cleaved with restriction endonuclease B, which again preferably leaves sticky ends. In the case of the DNA  
30 fragments from individual A, an oligonucleotide adaptor (#2), with complementary sticky ends for enzyme B, is ligated to the restriction fragments generated by cleavage with enzyme B.

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In step C, the DNA fragments from individual A are amplified using the polymerase chain reaction (PCR) with an oligonucleotide primer complementary to adaptor #1 and with a biotinylated oligonucleotide primer complementary to adaptor #2.

In step D, the amplified products originating from individual A are mixed with the non-amplified fragments of step B from individual B. The mixed DNA fragments are then heat denatured, annealed, and adsorbed onto an avidin-coated solid support (e.g., beads). The avidin coated support containing the adsorbed fragments is thoroughly washed. If desired, the adsorbed fragments may be eluted, re-amplified with the same primers as above, adsorbed onto a fresh avidin-containing support, and thoroughly washed. This step can be repeated as many times as is necessary or desired.

In step E, the fragments adsorbed to the avidin-coated beads are eluted and amplified using PCR with primers complementary to adaptor #3. The amplified products should correspond to the desired restriction fragments described above. These amplified fragments are cloned and then tested individually using the Southern DNA blot hybridization method for their ability to display the desired RFLP.

#### 25                    Other Embodiments

The above examples are, therefore, to be construed as merely illustrative, and not limitative of the remainder of the disclosure in any way whatsoever.

From the above description, one skilled in the art can easily ascertain the essential characteristics of the present invention, and without departing from the spirit and scope thereof, can make various changes and modifications of the invention to adapt it to various usages and conditions. All publications cited herein are

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fully incorporated by reference herein in their entirety.  
Other embodiments are in the claims set forth below.

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Claims

1. A method for detecting the presence or absence of a polymorphic restriction site in a nucleic acid, said method comprising the steps of:

- 5 (a) amplifying said nucleic acid by PCR using a first and a second primer flanking said polymorphic restriction site, said first primer being tagged with the first member of a specific binding pair, said second primer being tagged with a detectable label;
- 10 (b) digesting the PCR product of step (a) with the restriction endonuclease corresponding to said polymorphic restriction site;
- (c) contacting the reaction product of step (b) with the second member of said specific binding pair,
- 15 immobilized on a solid support; and
- (d) measuring the level of said detectable label bound to said solid support, the presence of said detectable label bound to said solid support being an indication of the absence of said polymorphic restriction
- 20 site in said nucleic acid.

2. A method for detecting the presence or absence of a polymorphic restriction site in a nucleic acid, said method comprising the steps of:

- (a) amplifying said nucleic acid by PCR using a
- 25 first and a second primer flanking said polymorphic restriction site, said first primer being tagged with the first member of a specific binding pair, said second primer being tagged with a first detectable label;
- (b) digesting the PCR product of step (a) with the
- 30 restriction endonuclease corresponding to said polymorphic restriction site;
- (c) annealing and ligating to the single-stranded ends generated in the reaction of step (b) an oligonucleotide tagged with a second detectable label;

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(d) contacting the reaction product of step (c) with the second member of said specific binding pair, immobilized on a solid support; and

(e) determining the levels of said first and  
5 second detectable labels bound to said solid support, the presence of only said first detectable label bound to said solid support being an indication of a homozygote lacking said polymorphic restriction site, the presence  
10 of only said second detectable label bound to said solid support being an indication of a homozygote containing said polymorphic restriction site, and the presence of both said first and second detectable labels bound to said solid support being an indication of a heterozygote.

3. A method for detecting the presence or absence  
15 of a polymorphic restriction site in a nucleic acid, said method comprising the steps of:

(a) amplifying said nucleic acid by PCR using a first and a second primer flanking said polymorphic restriction site, said first primer being tagged with a  
20 detectable label, said second primer being unlabeled;

(b) digesting a portion of the reaction of step (a) with the restriction endonuclease corresponding to said polymorphic restriction site, while leaving another portion of said reaction of step (a) undigested;

25 (c) denaturing said digested and undigested portions from step (b);

(d) contacting the product of step (c) with an oligonucleotide complementary to a sequence in the strand of said product of step (c) containing said detectable  
30 label, said sequence being between said polymorphic restriction and the sequence complementary to said second primer, said oligonucleotide being tagged with a first member of a specific binding pair;

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(e) contacting the reaction product of step (d) with the second member of said specific binding pair, immobilized on a solid support; and

(f) determining the ratio of the levels of said  
5 detectable label bound to said solid support between undigested and digested samples, a ratio of 1:0 between equivalent portions of said undigested and digested samples being an indication of a homozygote containing said polymorphic restriction site, a ratio of 1:1 between  
10 equivalent portions of said undigested and digested samples being an indication of a homozygote lacking said polymorphic restriction site, and a ratio of 2:1 between equivalent portions of said undigested and digested samples being an indication of a heterozygote.

15 4. A method for detecting the presence or absence of a polymorphic restriction site in a nucleic acid, said method comprising the steps of:

(a) amplifying said nucleic acid by PCR using a first and a second primer flanking said polymorphic  
20 restriction site, said first primer being tagged with a first detectable label, said second primer being tagged with a second detectable label;

(b) digesting the reaction product of step (a) with the restriction endonuclease corresponding to said  
25 polymorphic restriction site;

(c) denaturing the reaction product of step (b);

(d) contacting the product of step (c) with a first and a second oligonucleotide, said first oligonucleotide being complementary to a first sequence  
30 in the strand of said product of step (c) containing said first detectable label, said first sequence being between said polymorphic restriction site and the sequence corresponding to said first primer, said first oligonucleotide being tagged with the first member of a

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- first specific binding pair, said second oligonucleotide being complementary to a second sequence in the strand of said product of step (c) containing said second detectable label, said second sequence being on the same
- 5 side of said polymorphic restriction site as said first sequence, said second sequence not being contained within or being complementary to either of said first or second primers, said second oligonucleotide being tagged with the first member of a second specific binding pair;
- 10 (e) contacting a first portion of the reaction product of step (d) with the second member of said first specific binding pair, immobilized on a first solid support;
- (f) contacting a second portion of the reaction
- 15 product of step (d) with the second member of said second specific binding pair, immobilized on a second solid support; and
- (g) determining the ratio of the levels of said first and second detectable labels bound to said first
- 20 and second solid supports, a ratio of 1:0 between equivalent amounts of said first and second portions being an indication of a homozygote containing said polymorphic restriction site, a ratio of 1:1 between equivalent amounts of said first and second portions
- 25 being an indication of a homozygote lacking said polymorphic restriction site, and a ratio of 2:1 between equivalent amounts of said first and second portions being an indication of a heterozygote.

5. A method for detecting the presence or absence

30 of a polymorphic restriction site in a nucleic acid, said method comprising the steps of:

- (a) amplifying said nucleic acid by PCR using a first and a second primer flanking said polymorphic restriction site, said first primer being tagged with a

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first detectable label, said second primer being tagged with a second detectable label;

(b) digesting the reaction product of step (a) with the restriction endonuclease corresponding to said  
5 polymorphic restriction site;

(c) denaturing the reaction product of step (b);

(d) contacting the product of step (c) with a first and a second oligonucleotide, said first oligonucleotide being complementary to a first sequence  
10 in the strand of said product of step (c) containing said first detectable label, said first sequence being between said polymorphic restriction site and the sequence complementary to said second primer, said first oligonucleotide being tagged with the first member of a  
15 first specific binding pair, said second oligonucleotide being complementary to a second sequence in the strand of said product of step (c) containing said second detectable label, said second sequence being on the same side of said polymorphic restriction site as said first  
20 sequence, said second sequence not being contained within or being complementary to either of said first or second primers, said second oligonucleotide being tagged with the first member of a second specific binding pair;

(e) contacting a first portion of the reaction  
25 product of step (d) with the second member of said first specific binding pair, immobilized on a first solid support;

(f) contacting a second portion of the reaction product of step (d) with the second member of said second  
30 specific binding pair, immobilized on a second solid support; and

(g) determining the ratio of the levels of said first and second detectable labels bound to said first and second solid supports, a ratio of 0:1 between  
35 equivalent amounts of said first and second portions



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being an indication of a homozygote containing said polymorphic restriction site, a ratio of 1:1 between equivalent amounts of said first and second portions being an indication of a homozygote lacking said  
5 polymorphic restriction site, and a ratio of 1:2 between equivalent amounts of said first and second portions being an indication of a heterozygote.

6. A method for detecting the presence or absence of a polymorphic restriction site in a nucleic acid, said  
10 method comprising the steps of:

(a) amplifying said nucleic acid by PCR using a first and a second primer flanking said polymorphic restriction site, said first primer being tagged with a first detectable label, said second primer being tagged  
15 with a second detectable label;

(b) digesting the reaction product of step (a) with the restriction endonuclease corresponding to said polymorphic restriction site;

(c) denaturing the reaction product of step (b);

20 (d) contacting the product of step (c) with a first and a second oligonucleotide, said first oligonucleotide being complementary to a first sequence in the strand of said product of step (c) containing said first detectable label, said first sequence being between  
25 said polymorphic restriction site and the sequence corresponding to said first primer, said first oligonucleotide being tagged with the first member of a specific binding pair, said second oligonucleotide being complementary to a second sequence in the strand of said  
30 product of step (c) containing said second detectable label, said second sequence being on the same side of said polymorphic restriction site as said first sequence, said second sequence not being contained within or being complementary to either of said first or second primers,

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said second oligonucleotide being tagged with said first member of said specific binding pair;

(e) contacting the reaction product of step (d) with the second member of said specific binding pair,  
5 immobilized on a solid support; and

(f) determining the ratio of the levels of said first and second detectable labels bound to said solid support, a ratio of 1:0 being an indication of a homozygote containing said polymorphic restriction site,  
10 a ratio of 1:1 being an indication of a homozygote lacking said polymorphic restriction site, and a ratio of 2:1 being an indication of a heterozygote.

7. A method for detecting the presence or absence of a polymorphic restriction site in a nucleic acid, said  
15 method comprising the steps of:

(a) amplifying said nucleic acid by PCR using a first and a second primer flanking said polymorphic restriction site, said first primer being tagged with a first detectable label, said second primer being tagged  
20 with a second detectable label;

(b) digesting the reaction product of step (a) with the restriction endonuclease corresponding to said polymorphic restriction site;

(c) denaturing the reaction product of step (b);

25 (d) contacting the product of step (c) with a first and a second oligonucleotide, said first oligonucleotide being complementary to a first sequence in the strand of said product of step (c) containing said first detectable label, said first sequence being between  
30 said polymorphic restriction site and the sequence complementary to said second primer, said first oligonucleotide being tagged with the first member of a specific binding pair, said second oligonucleotide being complementary to a second sequence in the strand of said

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product of step (c) containing said second detectable label, said second sequence being on the same side of said polymorphic restriction site as said first sequence, said second sequence not being contained within or being  
5 complementary to either of said first or second primers, said second oligonucleotide being tagged with said first member of said specific binding pair;

(e) contacting the reaction product of step (d) with the second member of said specific binding pair,  
10 immobilized on a solid support; and

(f) determining the ratio of the levels of said first and second detectable labels bound to said solid support, a ratio of 0:1 being an indication of a homozygote containing said polymorphic restriction site,  
15 a ratio of 1:1 being an indication of a homozygote lacking said polymorphic restriction site, and a ratio of 1:2 being an indication of a heterozygote.

8. A method for detecting the presence or absence of a polymorphic restriction site in a nucleic acid, said  
20 method comprising the steps of:

(a) amplifying said nucleic acid by PCR using a first and a second primer flanking said polymorphic restriction site, said first primer being tagged with the first member of a first specific binding pair, said  
25 second primer being tagged with a detectable label;

(b) digesting the reaction product of step (a) with the restriction endonuclease corresponding to said polymorphic restriction site;

(c) contacting the reaction product of step (b)  
30 with the second member of said first specific binding pair, immobilized on a first solid support;

(d) denaturing the reaction product of step (c) not bound to said first solid support;

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(e) contacting the product of step (d) with an oligonucleotide complementary to a sequence in the strand of said product of step (d) containing said detectable label, said sequence being between said polymorphic  
5 restriction site and the sequence corresponding to said second primer, said oligonucleotide being tagged with the first member of a second specific binding pair;

(f) contacting the reaction product of step (e) with the second member of said second specific binding  
10 pair, immobilized on a second solid support; and

(g) determining the ratio of the level of said detectable label bound to said first solid support to the level of said detectable label bound to said second solid support, a ratio of 0:1 being an indication of a  
15 homozygote containing said polymorphic restriction site, in a case where the total amount of said reaction product from step (c) not bound to said first solid support was used in steps (d), (e), and (f); a ratio of 1:0 being an indication of a homozygote lacking said polymorphic  
20 restriction site, in a case where the total amount of said reaction product from step (c) not bound to said first solid support was used in steps (d), (e), and (f); and a ratio of 1:1 being an indication of a heterozygote, in a case where the total amount of said reaction product  
25 from step (c) not bound to said first solid support was used in steps (d), (e), and (f).

9. A method for detecting the presence or absence of a polymorphic restriction site in a nucleic acid, said method comprising the steps of:

30 (a) amplifying said nucleic acid by PCR using a first and a second primer flanking said polymorphic restriction site, said first primer being tagged with a detectable label, said second primer being unlabeled;

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(b) digesting the reaction product of step (a) with the restriction endonuclease corresponding to said polymorphic restriction site;

(c) annealing and ligating to the single-stranded  
5 ends generated in the reaction of step (b) a first oligonucleotide tagged with the first member of a first specific binding pair;

(d) contacting the reaction product of step (c) with the second member of said first specific binding  
10 pair, immobilized on a first solid support;

(e) denaturing the reaction product of step (d) not bound to said first solid support;

(f) contacting the product of step (e) with a second oligonucleotide complementary to a sequence in the  
15 strand of said product of step (e) containing said detectable label, said sequence being between said polymorphic restriction site and either the sequence corresponding to said first primer or the sequence complementary to said second primer, said second  
20 oligonucleotide being tagged with the first member of a second specific binding pair;

(g) contacting the reaction product of step (f) with the second member of said second specific binding pair, immobilized on a second solid support; and

25 (h) determining the ratio of the level of said detectable label bound to said first solid support to the level of said detectable label bound to said second solid support, a ratio of 1:0 being an indication of a homozygote containing said polymorphic restriction site,  
30 in a case where the total amount of said reaction product from step (d) not bound to said first solid support was used in steps (e), (f), and (g); a ratio of 0:1 being an indication of a homozygote lacking said polymorphic restriction site, in a case where the total amount of  
35 said reaction product from step (d) not bound to said

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first solid support was used in steps (e), (f), and (g);  
and a ratio of 1:1 being an indication of a heterozygote;  
in a case where the total amount of said reaction product  
from step (d) not bound to said first solid support was  
5 used in steps (e), (f), and (g).

10. A method for detecting the presence or  
absence of a polymorphic restriction site in a nucleic  
acid, said method comprising the steps of:

(a) amplifying said nucleic acid by PCR using a  
10 first and a second primer flanking said polymorphic  
restriction site, said first primer being tagged with the  
first member of a first specific binding pair, said  
second primer being tagged with a detectable label;

(b) digesting the reaction product of step (a)  
15 with the restriction endonuclease corresponding to said  
polymorphic restriction site;

(c) contacting the reaction product of step (b)  
with the second member of said first specific binding  
pair, immobilized on a first solid support;

20 (d) denaturing the reaction product from step (c)  
not bound to said first solid support;

(e) contacting the product of step (d) with an  
oligonucleotide complementary to a sequence in the strand  
of said product of step (d) containing said detectable  
25 label, said sequence being between said polymorphic  
restriction site and the sequence corresponding to said  
second primer, said oligonucleotide being immobilized on  
a second solid support; and

(f) determining the ratio of the level of said  
30 detectable label bound to said first solid support to the  
level of said detectable label bound to said second solid  
support, a ratio of 0:1 being an indication of a  
homozygote containing said polymorphic restriction site,  
in a case where the total amount of said reaction product

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from step (c) not bound to said first solid support was used in steps (d) and (e); a ratio of 1:0 being an indication of a homozygote lacking said polymorphic restriction site, in a case where the total amount of  
5 said reaction product from step (c) not bound to said first solid support was used in steps (d) and (e); and a ratio of 1:1 being an indication of a heterozygote, in a case where the total amount of said reaction product from step (c) not bound to said first solid support was used  
10 in steps (d) and (e).

11. A method for detecting the presence or absence of a polymorphic restriction site in a nucleic acid, said method comprising the steps of:

(a) amplifying said nucleic acid by PCR using a  
15 first and a second primer flanking said polymorphic restriction site, said first primer being tagged with a detectable label, said second primer being unlabeled;

(b) digesting the reaction product of step (a) with the restriction endonuclease corresponding to said  
20 polymorphic restriction site;

(c) annealing and ligating to the single-stranded ends generated in the reaction of step (b) a first oligonucleotide tagged with the first member of a first specific binding pair;

25 (d) contacting the reaction product of step (c) with the second member of said first specific binding pair, immobilized on a first solid support;

(e) denaturing the reaction product of step (d) not bound to said first solid support;

30 (f) contacting the product of step (e) with a second oligonucleotide complementary to a sequence in the strand of said product of step (e) containing said detectable label, said sequence being between said polymorphic restriction site and either the sequence

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corresponding to said first primer or the sequence complementary to said second primer, said second oligonucleotide being immobilized on a second solid support; and

- 5 (g) determining the ratio of the level of said detectable label bound to said first solid support to the level of said detectable label bound to said second solid support, a ratio of 1:0 being an indication of a homozygote containing said polymorphic restriction site,  
10 in a case where the total amount of said reaction product from step (d) not bound to said first solid support was used in steps (e) and (f); a ratio of 0:1 being an indication of a homozygote lacking said polymorphic restriction site, in a case where the total amount of  
15 said reaction product from step (d) not bound to said first solid support was used in steps (e) and (f); and a ratio of 1:1 being an indication of a heterozygote, in a case where the total amount of said reaction product from step (d) not bound to said first solid support was used  
20 in steps (e) and (f).

12. A method for detecting the presence or absence of a polymorphic restriction site in a nucleic acid, said method comprising the steps of:

- (a) amplifying said nucleic acid by PCR using a  
25 first and a second primer flanking said polymorphic restriction site, said first primer containing a first sequence not complementary to or present in said nucleic acid, said second primer containing a second sequence not complementary to or present in said nucleic acid;  
30 (b) amplifying the product of step (a) by PCR using a third and a fourth primer, said third primer containing said first sequence or a sequence complementary to said first sequence, said third primer being tagged with the first member of a specific binding



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pair, said fourth primer containing said second sequence or a sequence complementary to said second sequence, said fourth primer being tagged with a detectable label;

(c) digesting the product of step (b) with the  
5 restriction endonuclease corresponding to said polymorphic restriction site;

(d) contacting the reaction product of step (c) with the second member of said specific binding pair, immobilized on a solid support; and

10 (e) measuring the level of said detectable label bound to said solid support, the presence of said detectable label bound to said solid support being an indication of the absence of said polymorphic restriction site in said nucleic acid.

15 13. A method for detecting the presence or absence of a polymorphic restriction site in a nucleic acid, said method comprising the steps of:

(a) amplifying said nucleic acid by PCR using a first and a second primer flanking said polymorphic  
20 restriction site, said first primer containing a first sequence not complementary to or present in said nucleic acid, said second primer containing a second sequence not complementary to or present in said nucleic acid;

(b) amplifying the product of step (a) by PCR  
25 using a third and a fourth primer, said third primer containing said first sequence or a sequence complementary to said first sequence, said third primer being tagged with the first member of a specific binding pair, said fourth primer containing said second sequence  
30 or a sequence complementary to said second sequence, said fourth primer being tagged with a detectable label;

(c) digesting the PCR product of step (b) with the restriction endonuclease corresponding to said polymorphic restriction site;

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(d) annealing and ligating to the single-stranded ends generated in the reaction of step (c) an oligonucleotide tagged with a second detectable label;

(e) contacting the reaction product of step (d) with the second member of said specific binding pair, immobilized on a solid support; and

(f) determining the levels of said first and second detectable labels bound to said solid support, the presence of only said first detectable label bound to said solid support being an indication of a homozygote lacking said polymorphic restriction site, the presence of only said second detectable label bound to said solid support being an indication of a homozygote containing said polymorphic restriction site, and the presence of both said first and second detectable labels bound to said solid support being an indication of a heterozygote.

14. A method for detecting the presence or absence of a polymorphic restriction site in a nucleic acid, said method comprising the steps of:

(a) amplifying said nucleic acid by PCR using a first and a second primer flanking said polymorphic restriction site, said first primer containing a first sequence not complementary to or present in said nucleic acid;

(b) amplifying the product of step (a) by PCR using a third primer and said second primer, said third primer containing said first sequence, said third primer being tagged with a detectable label;

(c) digesting a portion of the reaction of step (b) with the restriction endonuclease corresponding to said polymorphic restriction site, while leaving another portion of said reaction of step (b) undigested;

(d) denaturing said digested and undigested portions from step (c);

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(e) contacting the product of step (d) with an oligonucleotide complementary to a second sequence in the strand of said product of step (d) containing said detectable label, said second sequence being between said polymorphic restriction site and the sequence complementary to said second primer, said oligonucleotide being tagged with a first member of a specific binding pair;

(f) contacting the reaction product of step (e) with the second member of said specific binding pair, immobilized on a solid support; and

(g) determining the ratio of the levels of said detectable label bound to said solid support between undigested and digested samples, a ratio of 1:0 between equivalent portions of said undigested and digested samples being an indication of a homozygote containing said polymorphic restriction site, a ratio of 1:1 between equivalent portions of said undigested and digested samples being an indication of a homozygote lacking said polymorphic restriction site, and a ratio of 2:1 between equivalent portions of said undigested and digested samples being an indication of a heterozygote.

15. A method for detecting the presence or absence of a polymorphic restriction site in a nucleic acid, said method comprising the steps of:

(a) amplifying said nucleic acid by PCR using a first and a second primer flanking said polymorphic restriction site, said first primer containing a first sequence not complementary to or present in said nucleic acid, said second primer containing a second sequence not complementary to or present in said nucleic acid;

(b) amplifying the product of step (a) by PCR using a third and a fourth primer, said third primer containing said first sequence or a sequence

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complementary to said first sequence, said third primer being tagged with a first detectable label, said fourth primer containing said second sequence or a sequence complementary to said second sequence, said fourth primer  
5 being tagged with a second detectable label;

(c) digesting the reaction product of step (b) with the restriction endonuclease corresponding to said polymorphic restriction site;

(d) denaturing the reaction product of step (c);

10 (e) contacting the product of step (d) with a first and a second oligonucleotide, said first oligonucleotide being complementary to a third sequence in the strand of said product of step (d) containing said first detectable label, said third sequence being between  
15 said polymorphic restriction site and the sequence corresponding to or complementary to said first primer, said first oligonucleotide being tagged with the first member of a first specific binding pair, said second oligonucleotide being complementary to a fourth sequence  
20 in the strand of said product of step (d) containing said second detectable label, said fourth sequence being on the same side of said polymorphic restriction site as said third sequence, said fourth sequence not being contained within or being complementary to any of said  
25 primers, said second oligonucleotide being tagged with the first member of a second specific binding pair;

(f) contacting a first portion of the reaction product of step (e) with the second member of said first specific binding pair, immobilized on a first solid  
30 support;

(g) contacting a second portion of the reaction product of step (e) with the second member of said second specific binding pair, immobilized on a second solid support; and

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(h) determining the ratio of the levels of said first and second detectable labels bound to said first and second solid supports, a ratio of 1:0 between equivalent amounts of said first and second portions  
5 being an indication of a homozygote containing said polymorphic restriction site, a ratio of 1:1 between equivalent amounts of said first and second portions being an indication of a homozygote lacking said polymorphic restriction site, and a ratio of 2:1 between  
10 equivalent amounts of said first and second portions being an indication of a heterozygote.

16. A method for detecting the presence or absence of a polymorphic restriction site in a nucleic acid, said method comprising the steps of:

15 (a) amplifying said nucleic acid by PCR using a first and a second primer flanking said polymorphic restriction site, said first primer containing a first sequence not complementary to or present in said nucleic acid, said second primer containing a second sequence not  
20 complementary to or present in said nucleic acid;

(b) amplifying the product of step (a) by PCR using a third and a fourth primer, said third primer containing said first sequence or a sequence complementary to said first sequence, said third primer  
25 being tagged with a first detectable label, said fourth primer containing said second sequence or a sequence complementary to said second sequence, said fourth primer being tagged with a second detectable label;

(c) digesting the reaction product of step (b)  
30 with the restriction endonuclease corresponding to said polymorphic restriction site;

(d) denaturing the reaction product of step (c);

(e) contacting the product of step (d) with a first and a second oligonucleotide, said first

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- oligonucleotide being complementary to a third sequence in the strand of said product of step (d) containing said first detectable label, said third sequence being between said polymorphic restriction site and the sequence
- 5 corresponding to or complementary to said second primer, said first oligonucleotide being tagged with the first member of a first specific binding pair, said second oligonucleotide being complementary to a fourth sequence in the strand of said product of step (d) containing said
- 10 second detectable label, said fourth sequence being on the same side of said polymorphic restriction site as said third sequence, said fourth sequence not being contained within or being complementary to any of said primers, said second oligonucleotide being tagged with
- 15 the first member of a second specific binding pair;
- (f) contacting a first portion of the reaction product of step (e) with the second member of said first specific binding pair, immobilized on a first solid support;
- 20 (g) contacting a second portion of the reaction product of step (e) with the second member of said second specific binding pair, immobilized on a second solid support; and
- (h) determining the ratio of the levels of said
- 25 first and second detectable labels bound to said first and second solid supports, a ratio of 0:1 between equivalent amounts of said first and second portions being an indication of a homozygote containing said polymorphic restriction site, a ratio of 1:1 between
- 30 equivalent amounts of said first and second portions being an indication of a homozygote lacking said polymorphic restriction site, and a ratio of 1:2 between equivalent amounts of said first and second portions being an indication of a heterozygote.

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17. A method for detecting the presence or absence of a polymorphic restriction site in a nucleic acid, said method comprising the steps of:

- (a) amplifying said nucleic acid by PCR using a  
5 first and a second primer flanking said polymorphic restriction site, said first primer containing a first sequence not complementary to or present in said nucleic acid, said second primer containing a second sequence not complementary to or present in said nucleic acid;
- 10 (b) amplifying the product of step (a) by PCR using a third and a fourth primer, said third primer containing said first sequence or a sequence complementary to said first sequence, said third primer being tagged with a first detectable label, said fourth  
15 primer containing said second sequence or a sequence complementary to said second sequence, said fourth primer being tagged with a second detectable label;
- (c) digesting the reaction product of step (b) with the restriction endonuclease corresponding to said  
20 polymorphic restriction site;
- (d) denaturing the reaction product of step (c);
- (e) contacting the product of step (d) with a first and a second oligonucleotide, said first  
oligonucleotide being complementary to a third sequence  
25 in the strand of said product of step (d) containing said first detectable label, said third sequence being between said polymorphic restriction site and the sequence corresponding to or complementary to said first primer, said first oligonucleotide being tagged with the first  
30 member of a specific binding pair, said second oligonucleotide being complementary to a fourth sequence in the strand of said product of step (d) containing said second detectable label, said fourth sequence being on the same side of said polymorphic restriction site as  
35 said third sequence, said fourth sequence not being

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contained within or being complementary to any of said primers, said second oligonucleotide being tagged with said first member of said specific binding pair;

(f) contacting the reaction product of step (e) with the second member of said specific binding pair, immobilized on a solid support; and

(g) determining the ratio of the levels of said first and second detectable labels bound to said solid support, a ratio of 1:0 being an indication of a homozygote containing said polymorphic restriction site, a ratio of 1:1 being an indication of a homozygote lacking said polymorphic restriction site, and a ratio of 2:1 being an indication of a heterozygote.

18. A method for detecting the presence or absence of a polymorphic restriction site in a nucleic acid, said method comprising the steps of:

(a) amplifying said nucleic acid by PCR using a first and a second primer flanking said polymorphic restriction site, said first primer containing a first sequence not complementary to or present in said nucleic acid, said second primer containing a second sequence not complementary to or present in said nucleic acid;

(b) amplifying the product of step (a) by PCR using a third and a fourth primer, said third primer containing said first sequence or a sequence complementary to said first sequence, said third primer being tagged with a first detectable label, said fourth primer containing said second sequence or a sequence complementary to said second sequence, said fourth primer being tagged with a second detectable label;

(c) digesting the reaction product of step (b) with the restriction endonuclease corresponding to said polymorphic restriction site;

(d) denaturing the reaction product of step (c);



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(e) contacting the product of step (d) with a first and a second oligonucleotide, said first oligonucleotide being complementary to a third sequence in the strand of said product of step (d) containing said first detectable label, said third sequence being between said polymorphic restriction site and the sequence corresponding to or complementary to said second primer, said first oligonucleotide being tagged with the first member of a specific binding pair, said second oligonucleotide being complementary to a fourth sequence in the strand of said product of step (d) containing said second detectable label, said fourth sequence being on the same side of said polymorphic restriction site as said third sequence, said fourth sequence not being contained within or being complementary to any of said primers, said second oligonucleotide being tagged with said first member of said specific binding pair;

(f) contacting the reaction product of step (e) with the second member of said specific binding pair, immobilized on a solid support; and

(g) determining the ratio of the levels of said first and second detectable labels bound to said solid support, a ratio of 0:1 being an indication of a homozygote containing said polymorphic restriction site, a ratio of 1:1 being an indication of a homozygote lacking said polymorphic restriction site, and a ratio of 1:2 being an indication of a heterozygote.

19. A method for detecting the presence or absence of a polymorphic restriction site in a nucleic acid, said method comprising the steps of:

(a) amplifying said nucleic acid by PCR using a first and a second primer flanking said polymorphic restriction site, said first primer containing a first sequence not complementary to or present in said nucleic

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acid, said second primer containing a second sequence not complementary to or present in said nucleic acid;

(b) amplifying the product of step (a) by PCR using a third and a fourth primer, said third primer  
5 containing said first sequence or a sequence complementary to said first sequence, said third primer being tagged with the first member of a first specific binding pair, said fourth primer containing said second  
10 sequence or a sequence complementary to said second sequence, said fourth primer being tagged with a detectable label;

(c) digesting the reaction product of step (b) with the restriction endonuclease corresponding to said polymorphic restriction site;

15 (d) contacting the reaction product of step (c) with the second member of said first specific binding pair, immobilized on a first solid support;

(e) denaturing the reaction product of step (d) not bound to said first solid support;

20 (f) contacting the product of step (e) with an oligonucleotide complementary to a third sequence in the strand of said product of step (e) containing said detectable label, said third sequence being between said polymorphic restriction site and the sequence  
25 corresponding to or complementary to said second primer, said oligonucleotide being tagged with the first member of a second specific binding pair;

(g) contacting the reaction product of step (f) with the second member of said second specific binding  
30 pair, immobilized on a second solid support; and

(h) determining the ratio of the level of said detectable label bound to said first solid support to the level of said detectable label bound to said second solid support, a ratio of 0:1 being an indication of a  
35 homozygote containing said polymorphic restriction site,

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in a case where the total amount of said reaction product from step (d) not bound to said first solid support was used in steps (e), (f), and (g); a ratio of 1:0 being an indication of a homozygote lacking said polymorphic restriction site, in a case where the total amount of said reaction product from step (d) not bound to said first solid support was used in steps (e), (f), and (g); and a ratio of 1:1 being an indication of a heterozygote, in a case where the total amount of said reaction product from step (d) not bound to said first solid support was used in steps (e), (f), and (g).

20. A method for detecting the presence or absence of a polymorphic restriction site in a nucleic acid, said method comprising the steps of:

15 (a) amplifying said nucleic acid by PCR using a first and a second primer flanking said polymorphic restriction site, said first primer containing a first sequence not complementary to or present in said nucleic acid;

20 (b) amplifying the product of step (a) by PCR using a third primer and said second primer, said third primer containing said first sequence, said third primer being tagged with a detectable label;

25 (c) digesting the reaction product of step (b) with the restriction endonuclease corresponding to said polymorphic restriction site;

(d) annealing and ligating to the single-stranded ends generated in the reaction of step (c) a first oligonucleotide tagged with the first member of a first specific binding pair;

30 (e) contacting the reaction product of step (d) with the second member of said first specific binding pair, immobilized on a first solid support;

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(f) denaturing the reaction product of step (e) not bound to said first solid support;

(g) contacting the product of step (f) with a second oligonucleotide complementary to a second sequence  
5 in the strand of said product of step (f) containing said detectable label, said second sequence being between said polymorphic restriction site and either the sequence corresponding to or complementary to said second primer or the sequence corresponding to or complementary to said  
10 first primer, said second oligonucleotide being tagged with the first member of a second specific binding pair;

(h) contacting the reaction product of step (g) with the second member of said second specific binding pair, immobilized on a second solid support; and

15 (i) determining the ratio of the level of said detectable label bound to said first solid support to the level of said detectable label bound to said second solid support, a ratio of 1:0 being an indication of a homozygote containing said polymorphic restriction site,  
20 in a case where the total amount of said reaction product from step (e) not bound to said first solid support was used in steps (f), (g), and (h); a ratio of 0:1 being an indication of a homozygote lacking said polymorphic restriction site, in a case where the total amount of  
25 said reaction product from step (e) not bound to said first solid support was used in steps (f), (g), and (h); and a ratio of 1:1 being an indication of a heterozygote; in a case where the total amount of said reaction product from step (e) not bound to said first solid support was  
30 used in steps (f), (g), and (h).

21. A method for detecting the presence or absence of a polymorphic restriction site in a nucleic acid, said method comprising the steps of:

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(a) amplifying said nucleic acid by PCR using a first and a second primer flanking said polymorphic restriction site, said first primer containing a first sequence not complementary to or present in said nucleic acid, said second primer containing a second sequence not complementary to or present in said nucleic acid;

(b) amplifying the product of step (a) by PCR using a third and a fourth primer, said third primer containing said first sequence or a sequence complementary to said first sequence, said third primer being tagged with the first member of a first specific binding pair, said fourth primer containing said second sequence or a sequence complementary to said second sequence, said fourth primer being tagged with a detectable label;

(c) digesting the reaction product of step (b) with the restriction endonuclease corresponding to said polymorphic restriction site;

(d) contacting the reaction product of step (c) with the second member of said first specific binding pair, immobilized on a first solid support;

(e) denaturing the reaction product from step (d) not bound to said first solid support;

(f) contacting the product of step (e) with an oligonucleotide complementary to a third sequence in the strand of said product of step (e) containing said detectable label, said third sequence being between said polymorphic restriction site and the sequence corresponding to or complementary to said second primer, said oligonucleotide being immobilized on a second solid support; and

(g) determining the ratio of the level of said detectable label bound to said first solid support to the level of said detectable label bound to said second solid support, a ratio of 0:1 being an indication of a

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homozygote containing said polymorphic restriction site, in a case where the total amount of said reaction product from step (d) not bound to said first solid support was used in steps (e) and (f); a ratio of 1:0 being an indication of a homozygote lacking said polymorphic restriction site, in a case where the total amount of said reaction product from step (d) not bound to said first solid support was used in steps (e) and (f); and a ratio of 1:1 being an indication of a heterozygote, in a case where the total amount of said reaction product from step (d) not bound to said first solid support was used in steps (e) and (f).

22. A method for detecting the presence or absence of a polymorphic restriction site in a nucleic acid, said method comprising the steps of:

(a) amplifying said nucleic acid by PCR using a first and a second primer flanking said polymorphic restriction site, said first primer containing a first sequence not complementary to or present in said nucleic acid;

(b) amplifying the product of step (a) by PCR using a third primer and said second primer, said third primer containing said first sequence, said third primer being tagged with a detectable label;

(c) digesting the reaction product of step (b) with the restriction endonuclease corresponding to said polymorphic restriction site;

(d) annealing and ligating to the single-stranded ends generated in the reaction of step (c) a first oligonucleotide tagged with the first member of a first specific binding pair;

(e) contacting the reaction product of step (d) with the second member of said first specific binding pair, immobilized on a first solid support;

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(f) denaturing the reaction product of step (e) not bound to said first solid support;

(g) contacting the product of step (f) with a second oligonucleotide complementary to a second sequence  
5 in the strand of said product of step (f) containing said detectable label, said second sequence being between said polymorphic restriction site and either the sequence corresponding to or complementary to said second primer or the sequence corresponding to or complementary to said  
10 first primer, said second oligonucleotide being immobilized on a second solid support; and

(h) determining the ratio of the level of said detectable label bound to said first solid support to the level of said detectable label bound to said second solid  
15 support, a ratio of 1:0 being an indication of a homozygote containing said polymorphic restriction site, in a case where the total amount of said reaction product from step (e) not bound to said first solid support was used in steps (f) and (g); a ratio of 0:1 being an  
20 indication of a homozygote lacking said polymorphic restriction site, in a case where the total amount of said reaction product from step (e) not bound to said first solid support was used in steps (f) and (g); and a ratio of 1:1 being an indication of a heterozygote, in a  
25 case where the total amount of said reaction product from step (e) not bound to said first solid support was used in steps (f) and (g).

23. A kit for detecting the presence or absence of a polymorphic restriction site in a nucleic acid, said  
30 kit comprising one or more sets of a first and a second primer flanking said polymorphic restriction site, said first primer being tagged with the first member of a specific binding pair, said second primer being tagged with a detectable label.

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24. The kit of claim 23, wherein said kit further comprises the second member of said specific binding pair, immobilized on a solid support.

25. The kit of claim 23, wherein said kit further  
5 comprises an oligonucleotide complementary to the single-stranded ends generated in said nucleic acid upon digestion of said nucleic acid with the restriction enzyme corresponding to said polymorphic restriction site, said oligonucleotide being tagged with a second  
10 detectable label.

26. A kit for detecting the presence or absence of a polymorphic restriction site in a nucleic acid, said kit comprising:

(a) a first and a second primer flanking said  
15 polymorphic restriction site, said first primer being tagged with a detectable label, said second primer being unlabeled;

(b) an oligonucleotide complementary to a sequence in the strand of said nucleic acid complementary to said  
20 second primer, said sequence being between said polymorphic restriction site and the sequence complementary to said second primer, said oligonucleotide being tagged with a first member of a specific binding pair; and

25 (c) the second member of said specific binding pair, immobilized on a solid support.

27. A kit for detecting the presence or absence of a polymorphic restriction site in a nucleic acid, said kit comprising:

30 (a) a first and a second primer flanking said polymorphic restriction site, said first primer being



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tagged with a first detectable label, said second primer being tagged with a second detectable label;

(b) a first oligonucleotide, said first oligonucleotide being complementary to a first sequence  
5 in the strand of said nucleic acid complementary to said second primer, said first sequence being between said polymorphic restriction site and either the sequence corresponding to said first primer or the sequence complementary to said second primer, said first  
10 oligonucleotide being tagged with the first member of a first specific binding pair;

(c) a second oligonucleotide, said second oligonucleotide being complementary to a second sequence in the strand of said nucleic acid complementary to said  
15 first primer, said second sequence being on the same side of said polymorphic restriction site as said first sequence, said second sequence not being contained within or being complementary to either of said first or second primers, said second oligonucleotide being tagged with  
20 the first member of a second specific binding pair;

(d) the second member of said first specific binding pair, immobilized on a first solid support; and

(e) the second member of said second specific binding pair, immobilized on a second solid support.

25           28. The kit of claim 27, wherein said first and said second specific binding pairs are identical, and said first and said second solid supports are identical.

29. A kit for detecting the presence or absence of a polymorphic restriction site in a nucleic acid, said  
30 kit comprising:

(a) a first and a second primer flanking said polymorphic restriction site, said first primer being tagged with the first member of a first specific binding

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pair, said second primer being tagged with a detectable label;

(b) the second member of said first specific binding pair, immobilized on a first solid support;

5 (c) an oligonucleotide complementary to a first sequence in the strand of said nucleic acid containing the sequence corresponding to said second primer, said first sequence being between said polymorphic restriction site and said sequence corresponding to said second  
10 primer, said oligonucleotide being tagged with the first member of a second specific binding pair; and

(d) the second member of said second specific binding pair, immobilized on a second solid support.

30. A kit for detecting the presence or absence  
15 of a polymorphic restriction site in a nucleic acid, said kit comprising:

(a) a first and a second primer flanking said polymorphic restriction site, said first primer being tagged with a detectable label, said second primer being  
20 unlabeled; (b) a first oligonucleotide complementary to the single-stranded ends generated in said nucleic acid upon digestion of said nucleic acid with the restriction enzyme corresponding to said polymorphic restriction site, said oligonucleotide being tagged with the first  
25 member of a first specific binding pair;

(c) the second member of said first specific binding pair, immobilized on a first solid support;

(d) a second oligonucleotide complementary to a sequence in the strand of said nucleic acid complementary  
30 to said second primer, said sequence being between said polymorphic restriction site and either the sequence corresponding to said first primer or the sequence complementary to said second primer, said second

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oligonucleotide being tagged with the first member of a second specific binding pair; and

(e) the second member of said second specific binding pair, immobilized on a second solid support.

5           31. A kit for detecting the presence or absence of a polymorphic restriction site in a nucleic acid, said kit comprising:

          (a) a first and a second primer flanking said polymorphic restriction site, said first primer being  
10 tagged with the first member of a first specific binding pair, said second primer being tagged with a detectable label;

          (b) the second member of said first specific binding pair, immobilized on a first solid support; and

15           (c) an oligonucleotide complementary to a first sequence in the strand of said nucleic acid containing the sequence corresponding to said second primer, said first sequence being between said polymorphic restriction site and said sequence corresponding to said second  
20 primer, said oligonucleotide being immobilized on a second solid support.

          32. A kit for detecting the presence or absence of a polymorphic restriction site in a nucleic acid, said kit comprising:

25           (a) a first and a second primer flanking said polymorphic restriction site, said first primer being tagged with a detectable label, said second primer being unlabeled;

          (b) a first oligonucleotide complementary to the  
30 single-stranded ends generated in said nucleic acid upon digestion of said nucleic acid with the restriction enzyme corresponding to said polymorphic restriction

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site, said oligonucleotide being tagged with the first member of a first specific binding pair;

(c) the second member of said first specific binding pair, immobilized on a first solid support; and

5 (d) a second oligonucleotide complementary to a sequence in the strand of said nucleic acid complementary to said second primer, said sequence being between said polymorphic restriction site and either the sequence corresponding to said first primer or the sequence  
10 complementary to said second primer, said second oligonucleotide being immobilized on a second solid support.

33. A kit for detecting the presence or absence of a polymorphic restriction site in a nucleic acid, said  
15 kit comprising:

(a) a first and a second primer flanking said polymorphic restriction site, said first primer containing a first sequence not complementary to or present in said nucleic acid, said second primer  
20 containing a second sequence not complementary to or present in said nucleic acid;

(b) a third and a fourth primer, said third primer containing said first sequence or a sequence complementary to said first sequence, said third primer  
25 being tagged with the first member of a specific binding pair, said fourth primer containing said second sequence or a sequence complementary to said second sequence, said fourth primer being tagged with a detectable label.

34. The kit of claim 33, wherein said kit further  
30 comprises the second member of said specific binding pair, immobilized on a solid support.

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35. The kit of claim 33, wherein said kit further comprises an oligonucleotide complementary to the single-stranded ends generated in said nucleic acid upon digestion of said nucleic acid with the restriction enzyme corresponding to said polymorphic restriction site, said oligonucleotide being tagged with a second detectable label.

36. A kit for detecting the presence or absence of a polymorphic restriction site in a nucleic acid, said kit comprising:

- (a) a first and a second primer flanking said polymorphic restriction site, said first primer containing a first sequence not complementary to or present in said nucleic acid;
- 15 (b) a third primer containing said first sequence, said third primer being tagged with a detectable label;
- (c) an oligonucleotide complementary to a second sequence in the strand of said nucleic acid containing the sequence complementary to said second primer, said second sequence being between said polymorphic restriction site and said sequence complementary to said second primer, said oligonucleotide being tagged with a first member of a specific binding pair; and
- 20 (d) the second member of said specific binding pair, immobilized on a solid support.

37. A kit for detecting the presence or absence of a polymorphic restriction site in a nucleic acid, said kit comprising:

- (a) a first and a second primer flanking said polymorphic restriction site, said first primer containing a first sequence not complementary to or present in said nucleic acid, said second primer
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containing a second sequence not complementary to or present in said nucleic acid;

- (b) a third and a fourth primer, said third primer containing said first sequence or a sequence  
5 complementary to said first sequence, said third primer being tagged with a first detectable label, said fourth primer containing said second sequence or a sequence complementary to said second sequence, said fourth primer being tagged with a second detectable label;
- 10 (c) a first oligonucleotide, said first oligonucleotide being complementary to a third sequence in the strand of said nucleic acid complementary to said second primer, said third sequence being between said polymorphic restriction site and either the sequence  
15 complementary to said second primer or the sequence corresponding to said first primer, said first oligonucleotide being tagged with the first member of a first specific binding pair,
- (d) a second oligonucleotide, said second  
20 oligonucleotide being complementary to a fourth sequence in the strand of said nucleic acid complementary to said first primer, said fourth sequence being on the same side of said polymorphic restriction site as said third sequence, said fourth sequence not being contained within  
25 or being complementary to any of said primers, said second oligonucleotide being tagged with the first member of a second specific binding pair;
- (e) the second member of said first specific binding pair, immobilized on a first solid support; and  
30 (f) the second member of said second specific binding pair, immobilized on a second solid support.

38. The kit of claim 32, wherein said first and said second specific binding pairs are identical, and said first and said second solid supports are identical.

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39. A kit for detecting the presence or absence of a polymorphic restriction site in a nucleic acid, said kit comprising:

- 5 (a) a first and a second primer flanking said polymorphic restriction site, said first primer containing a first sequence not complementary to or present in said nucleic acid, said second primer containing a second sequence not complementary to or present in said nucleic acid;
- 10 (b) a third and a fourth primer, said third primer containing said first sequence or a sequence complementary to said first sequence, said third primer being tagged with the first member of a first specific binding pair, said fourth primer containing said second  
15 sequence or a sequence complementary to said second sequence, said fourth primer being tagged with a detectable label;
- (c) the second member of said first specific binding pair, immobilized on a first solid support;
- 20 (d) an oligonucleotide complementary to a third sequence in the strand of said nucleic acid corresponding to said second primer, said sequence being between said polymorphic restriction site and the sequence corresponding to said second primer, said oligonucleotide  
25 being tagged with the first member of a second specific binding pair; and
- (e) the second member of said second specific binding pair, immobilized on a second solid support.

40. A kit for detecting the presence or absence  
30 of a polymorphic restriction site in a nucleic acid, said kit comprising:

- (a) a first and a second primer flanking said polymorphic restriction site, said first primer

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containing a first sequence not complementary to or present in said nucleic acid;

(b) a third primer containing said first sequence, said third primer being tagged with a detectable label;

5 (c) a first oligonucleotide complementary to the single-stranded ends generated in said nucleic acid upon digestion of said nucleic acid with the restriction enzyme corresponding to said polymorphic restriction site, said oligonucleotide being tagged with the first  
10 member of a first specific binding pair;

(d) the second member of said first specific binding pair, immobilized on a first solid support;

(e) a second oligonucleotide complementary to a second sequence in the strand of said nucleic acid  
15 corresponding to said first primer, said second sequence being between said polymorphic restriction site and either the sequence complementary to said second primer or the sequence corresponding to said first primer, said second oligonucleotide being tagged with the first member  
20 of a second specific binding pair; and

(f) the second member of said second specific binding pair, immobilized on a second solid support.

41. A kit for detecting the presence or absence of a polymorphic restriction site in a nucleic acid, said  
25 kit comprising:

(a) a first and a second primer flanking said polymorphic restriction site, said first primer containing a first sequence not complementary to or present in said nucleic acid, said second primer  
30 containing a second sequence not complementary to or present in said nucleic acid;

(b) a third and a fourth primer, said third primer containing said first sequence or a sequence complementary to said first sequence, said third primer



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being tagged with the first member of a first specific binding pair, said fourth primer containing said second sequence or a sequence complementary to said second sequence, said fourth primer being tagged with a  
5 detectable label;

(c) the second member of said first specific binding pair, immobilized on a first solid support; and

(d) an oligonucleotide complementary to a third sequence in the strand of said nucleic acid corresponding  
10 to said second primer, said third sequence being between said polymorphic restriction site and the sequence corresponding to said second primer, said oligonucleotide being immobilized on a second solid support.

42. A kit for detecting the presence or absence  
15 of a polymorphic restriction site in a nucleic acid, said kit comprising:

(a) a first and a second primer flanking said polymorphic restriction site, said first primer containing a first sequence not complementary to or  
20 present in said nucleic acid;

(b) a third primer containing said first sequence, said third primer being tagged with a detectable label;

(c) a first oligonucleotide complementary to the single-stranded ends generated in said nucleic acid upon  
25 digestion of said nucleic acid with the restriction enzyme corresponding to said polymorphic restriction site, said oligonucleotide being tagged with the first member of a first specific binding pair;

(d) the second member of said first specific  
30 binding pair, immobilized on a first solid support; and

(e) a second oligonucleotide complementary to a second sequence in the strand of said nucleic acid corresponding to said first primer, said sequence being between said polymorphic restriction site and either the

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sequence corresponding to or complementary to said second primer or the sequence corresponding to or complementary to said first primer, said second oligonucleotide being immobilized on a second solid support.

- 5           43. A method for detecting the presence or absence of a polymorphic restriction site in a nucleic acid, said method comprising the steps of:
- (a) amplifying said nucleic acid by PCR using a first and a second primer flanking said polymorphic  
10 restriction site, whereby the resultant PCR product is of a defined size readily resolved by gel electrophoresis;
- (b) digesting the PCR product of step (a) with the restriction endonuclease corresponding to said  
polymorphic restriction site, said digestion products  
15 being differentially sized;
- (c) separating the reaction products of step (b) by gel electrophoresis; and
- (d) detecting said separated reaction products, the presence of only uncleaved products being an  
20 indication of a homozygote lacking said polymorphic restriction site, the presence of only cleaved products being an indication of a homozygote containing said polymorphic restriction site, and the presence of both  
cleaved and uncleaved products being an indication of a  
25 heterozygote.

44. The method of claim 43, wherein one or both of said first and second primers are tagged with a detectable label.

45. The method of claim 44, wherein said PCR  
30 product is 100-1000 base pairs in length.

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46. A kit for detecting the presence or absence of a polymorphic restriction site in a nucleic acid, said kit comprising: a first and a second primer flanking said polymorphic restriction site and capable of generating a  
5 PCR product of a defined size that is readily resolved by gel electrophoresis.

47. The kit of claim 46, wherein said first and/or said second primers are detectably labeled.

48. The kit of claim 46, wherein said PCR product  
10 generated is between 100 and 1000 base pairs in length.

49. The kit of claims 23, 26-27, 29-33, 36-37, 39-42, and 46, wherein multiple polymorphic restriction sites are detected.

50. A method for identifying a polymorphic  
15 restriction site in a nucleic acid, said method comprising the steps of:

(a) digesting DNA isolated from a first sample with a first restriction endonuclease;

(b) ligating to each of the ends of the reaction  
20 products of step (a) a first adaptor;

(c) digesting the products of step (b) with a second restriction endonuclease;

(d) ligating to each of the ends of the reaction products generated in step (c) a second adaptor;

25 (e) amplifying said reaction products of step (d) by PCR using a first primer complementary to said first adaptor and a second primer complementary to said second adaptor, said second primer being tagged with a first member of a specific binding pair;

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(f) in a separate set of reactions, digesting DNA isolated from a second sample with said first restriction endonuclease;

(g) ligating to each of the ends of the reaction products of step (f) a third adaptor;

(h) digesting the products of step (g) with said second restriction endonuclease;

(i) denaturing the products of step (e) and the products of step (h);

(j) combining the denatured products of step (i) under conditions allowing hybridization;

(k) contacting the hybridization products of step (j) with the second member of said specific binding pair, said second member being immobilized on a solid support;

(l) recovering said hybridization products captured on said solid support; and

(m) amplifying said products obtained in step (l) by PCR using a primer complementary to said third adaptor, an amplified product being an indication of a polymorphic restriction site corresponding to said second restriction endonuclease.

51. The method of claim 50, wherein said specific binding pair is avidin and biotin.

52. A kit for identifying a polymorphic restriction site in a nucleic acid, said kit comprising:

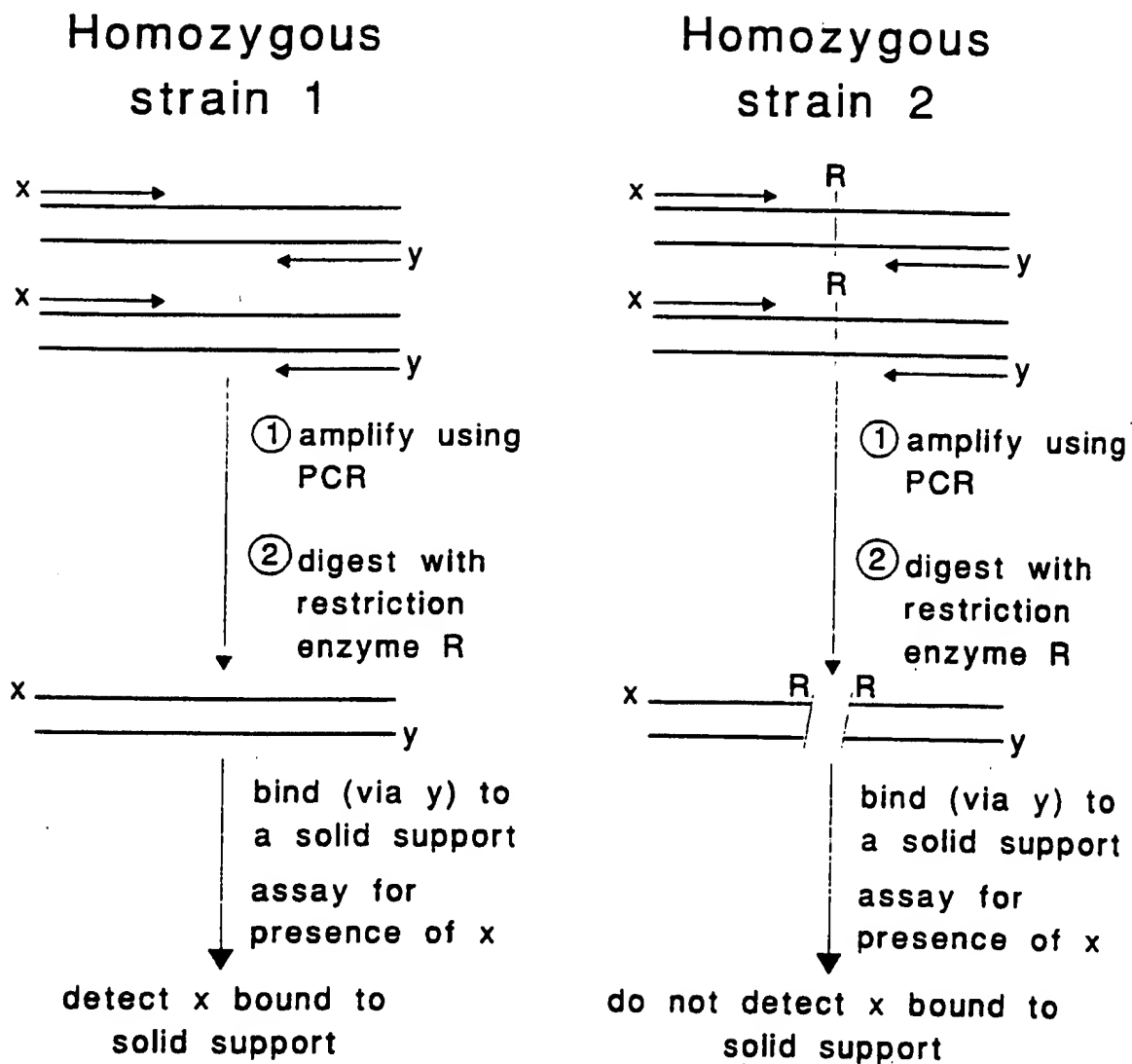
(a) a first DNA adaptor, a second DNA adaptor, and a third DNA adaptor, said first and third DNA adaptors having regions complementary to the ends generated by a first restriction endonuclease ends but differing in overall sequence and said second DNA adaptor having a region complementary to the ends generated by a second restriction endonuclease, said second restriction

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endonuclease site corresponding to said polymorphic restriction site; and

(b) a first primer, a second primer, and a third primer, said first primer being complementary to said first DNA adaptor, said second primer being complementary to said second DNA adaptor and being tagged with a first member of a specific binding pair, and said third primer being complementary to said third DNA adaptor.

53. The kit of claim 52, wherein said kit further comprises the second member of said specific binding pair immobilized on a solid support.

**FIG. 1**

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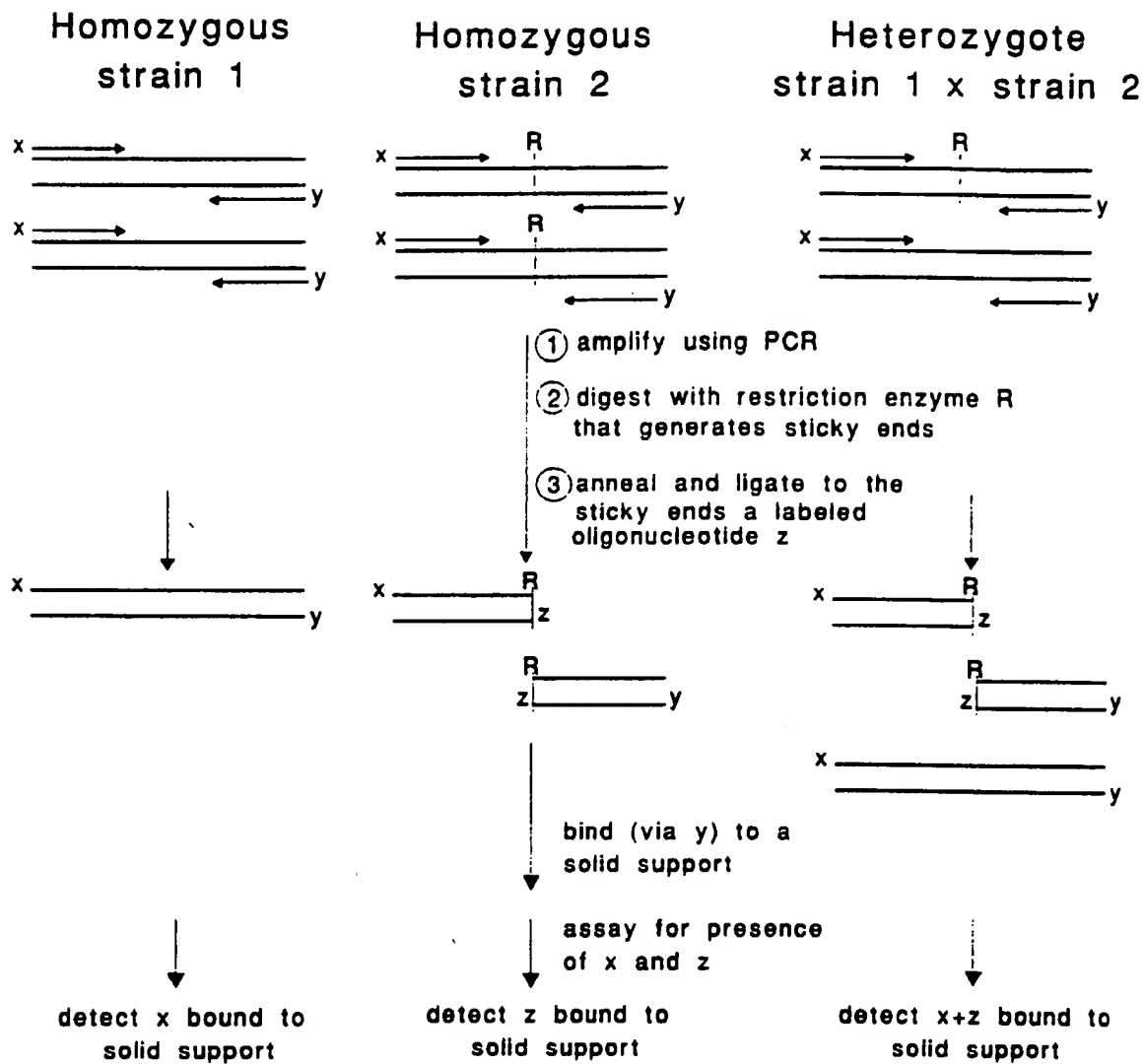


FIG. 2

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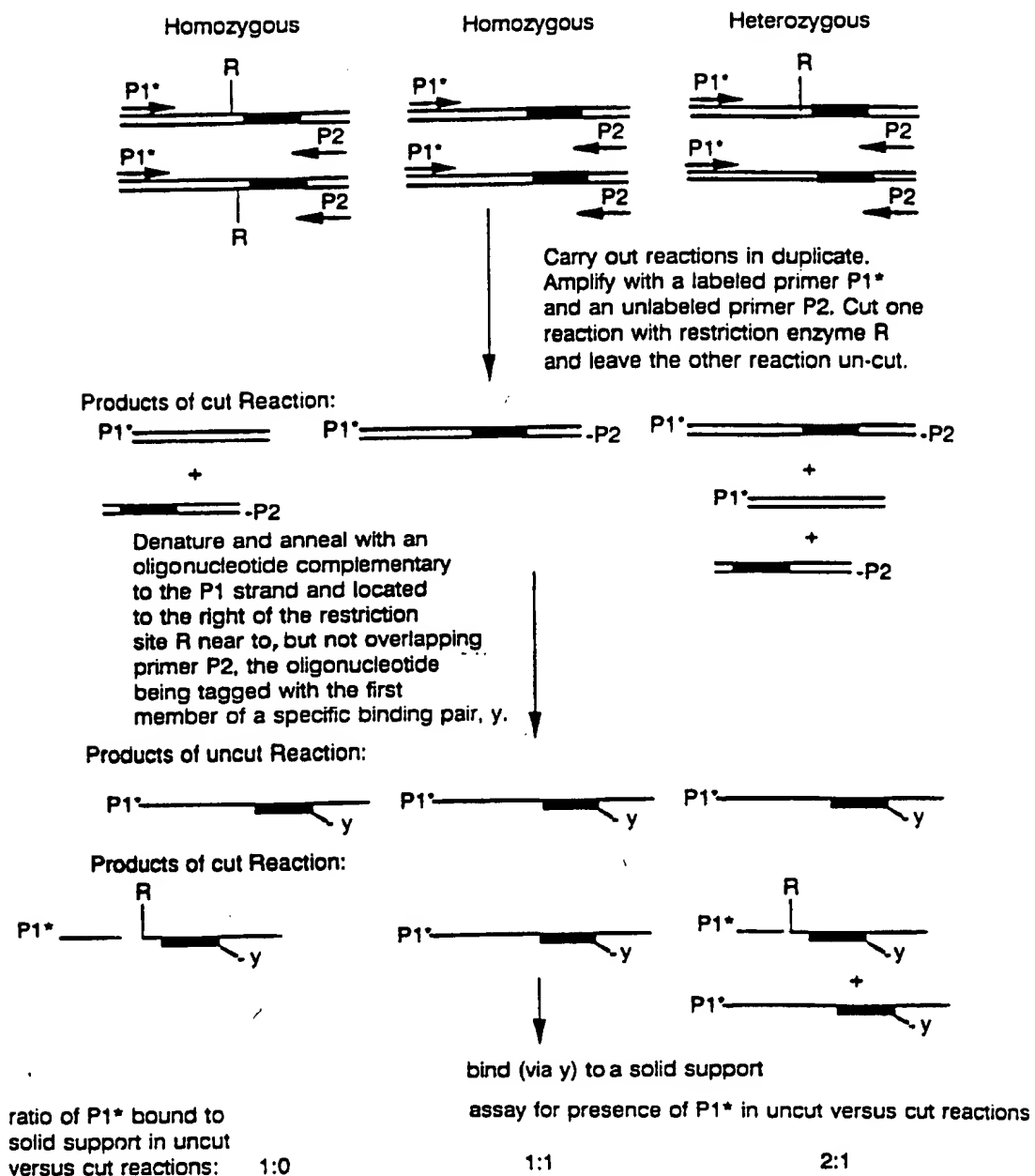
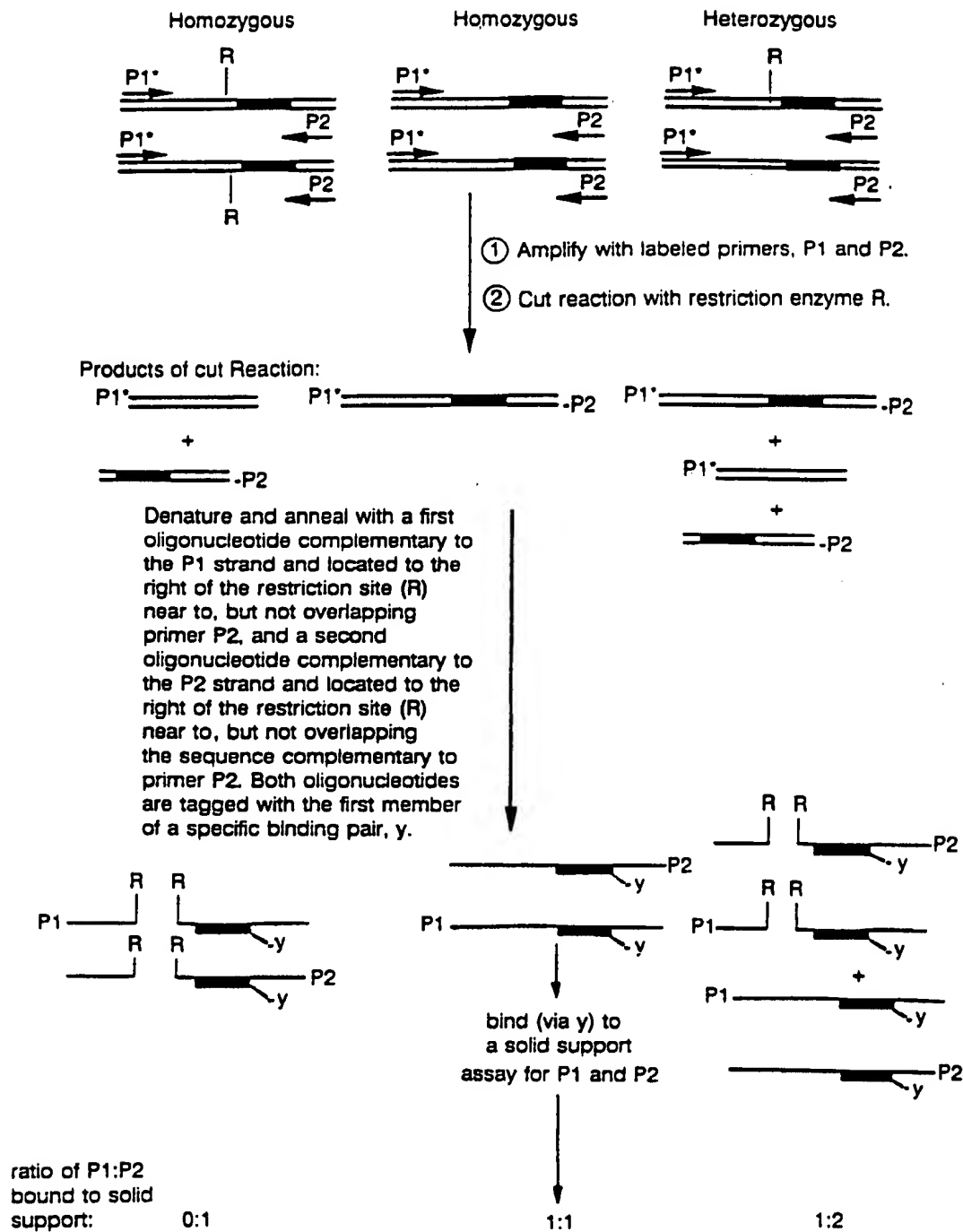


FIG. 3

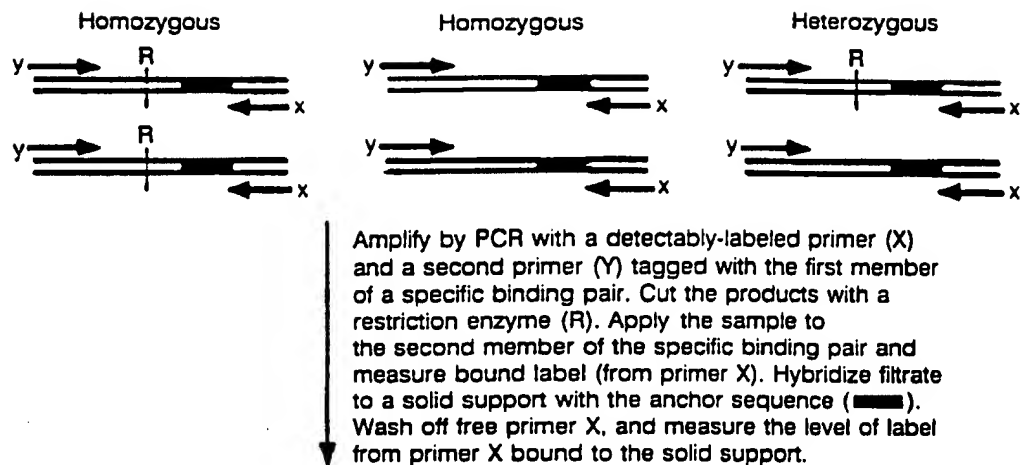


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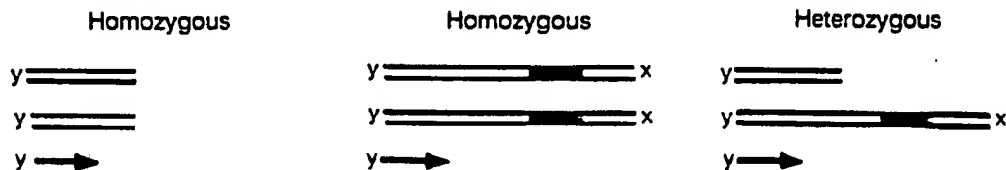


**FIG. 4**

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### Material bound to second member of specific binding pair

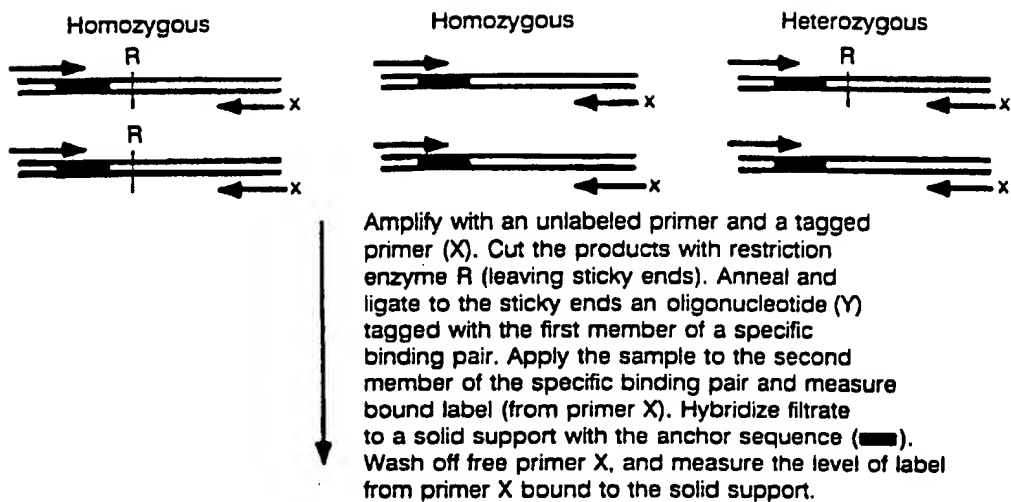


### Material bound to anchor sequence

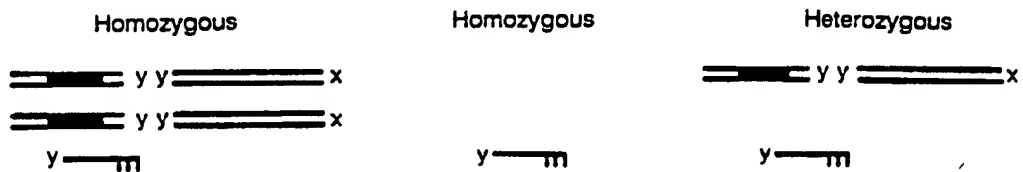


FIG. 5

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### Material bound to second member of specific binding pair



### Material bound to anchor sequence



FIG. 6

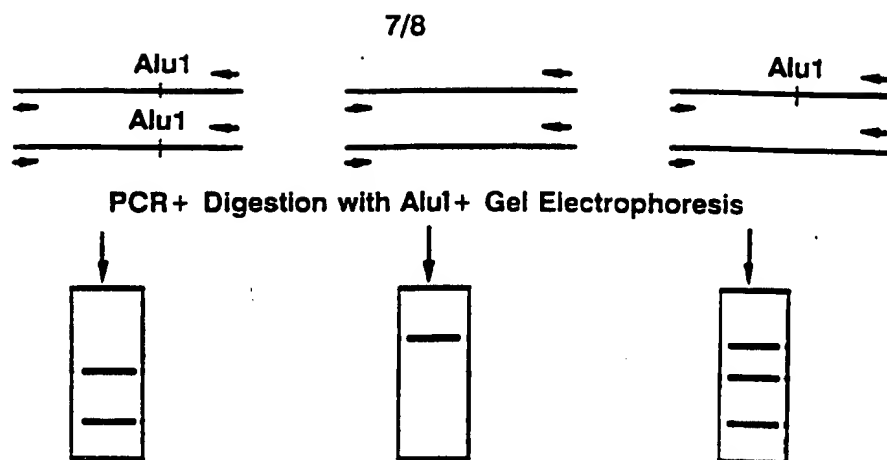


FIG. 7

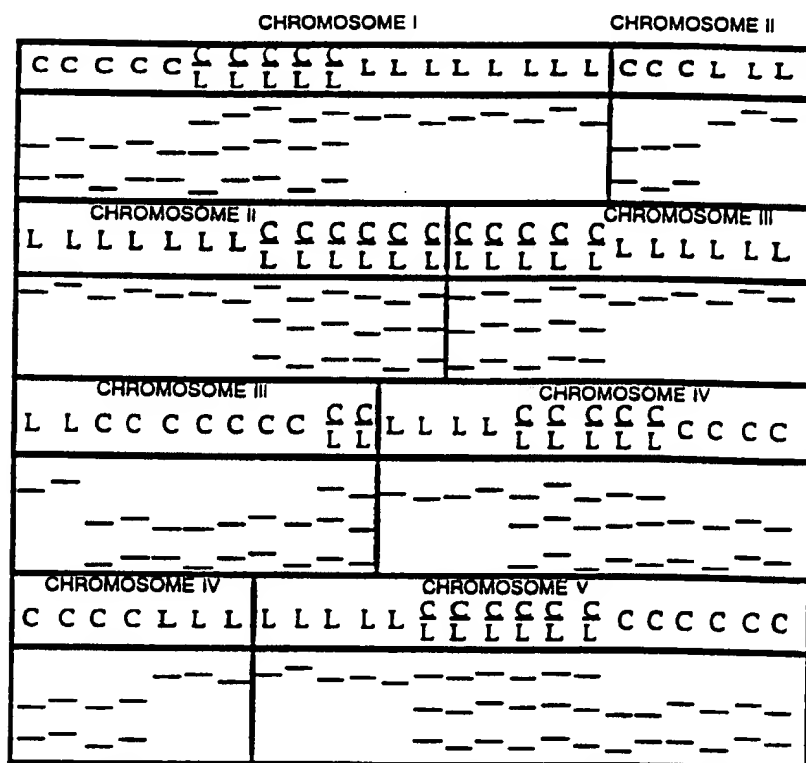
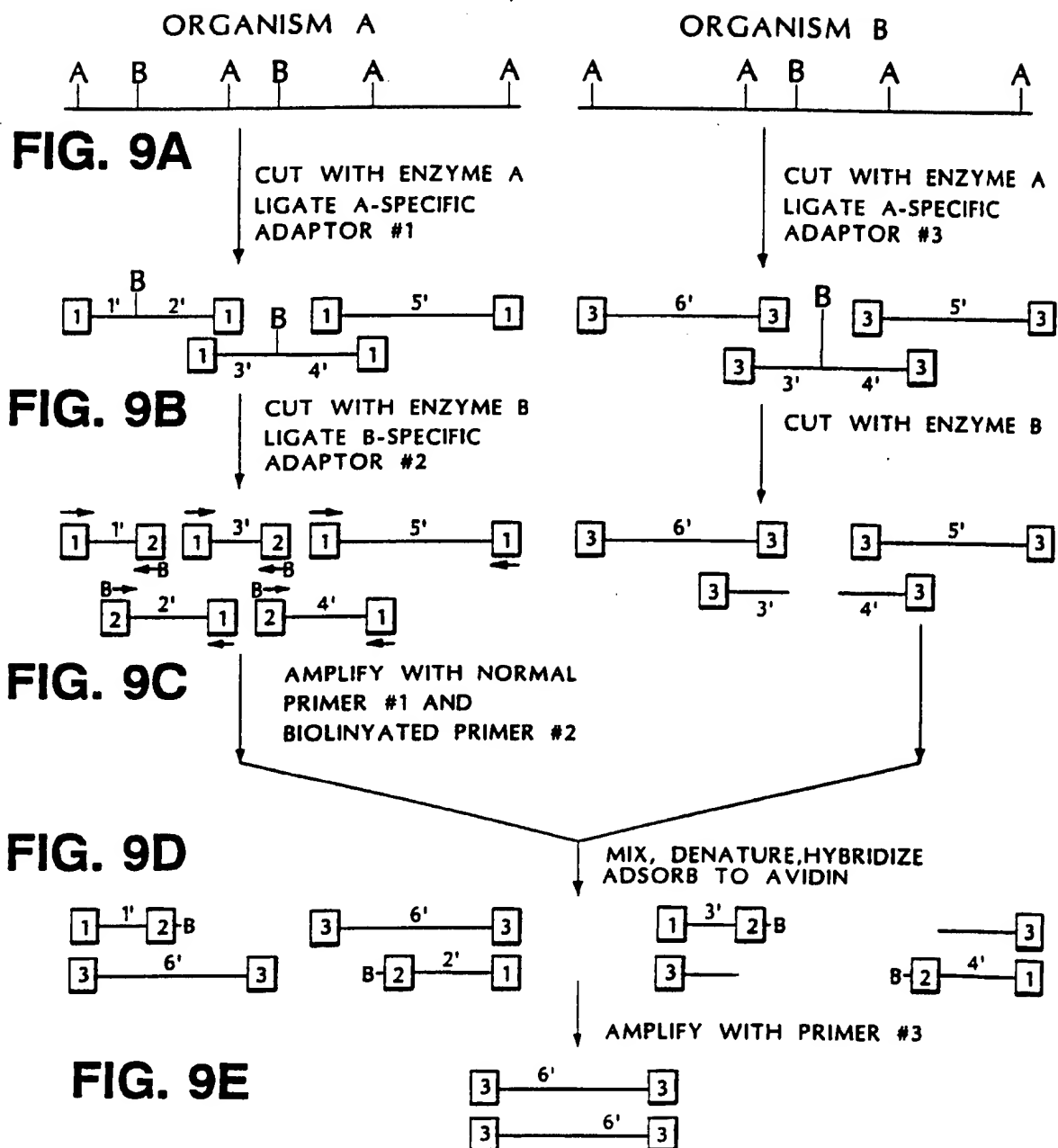


FIG. 8

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# INTERNATIONAL SEARCH REPORT

International application No.  
PCT/US95/03419

## A. CLASSIFICATION OF SUBJECT MATTER

IPC(6) : Please See Extra Sheet.

US CL : Please See Extra Sheet.

According to International Patent Classification (IPC) or to both national classification and IPC

## B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 424/94.1; 435/5, 6, 91.2, 810; 436/501, 518; 536/24.1, 24.33, 26.6, 204/182.8

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

Please See Extra Sheet.

## C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	US, A, 5,118,605 (URDEA ET AL) 02 June 1992, col. 2, lines 29-43.	1-42, 49
Y	US, A, 5,200,314 (URDEA ET AL) 06 April 1993, abstract, lines 1-14, col. 7, lines 39-65, and col. 8, lines 30-36.	1-42, 49
Y	US, A, 5,192,659 (SIMONS ET AL) 09 March 1993, col.4, lines 28-50.	43-48
Y	US, A, 5,294,534 (DATTAGUPTA ET AL) 15 March 1994, abstract, lines 1-24, and col. 2, lines 38-63 .	9, 15-20, 22, 30, 32, 37, 38, 41, 49-53

☒ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

* Special categories of cited documents:	* T	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
* A		document defining the general state of the art which is not considered to be of particular relevance
* E		earlier document published on or after the international filing date
* L		document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
* O		document referring to an oral disclosure, use, exhibition or other means
* P		document published prior to the international filing date but later than the priority date claimed
	* X	document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
	* Y	document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
	* Z	document member of the same patent family

Date of the actual completion of the international search

14 JUNE 1995

Date of mailing of the international search report

05 JUL 1995

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## INTERNATIONAL SEARCH REPORT

International application No.  
PCT/US95/03419

## C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y, P	Nucleic Acids Research, Volume 22, No. 24, issued 11 December 1994, Z. Guo et al, "Direct fluorescence analysis of genetic polymorphisms by hybridization with oligonucleotide arrays on glass supports", pages 5456-5465, especially abstract, and page 5456, col. 2, paragraph 3, lines 1-14.	1-42, 49
Y	Science, Volume 259, issued 12 February 1993, Lisitsyn et al, "Cloning the Differences Between Two Complex Genomes", pages 946-951, especially page 947, Figure 1.	50-53

# INTERNATIONAL SEARCH REPORT

International application No.  
PCT/US95/03419

**A. CLASSIFICATION OF SUBJECT MATTER:**  
**IPC (6):**

A61K 38/43; C12Q 1/68, 1/70; C12P 19/34; G01N 27/26, 33/53; C07H 21/04; C07K 17/00, 17/02, 17/06

**A. CLASSIFICATION OF SUBJECT MATTER:**  
**US CL :**

424/94.1; 435/5, 6, 91.2, 810; 436/501, 518; 536/24.1, 24.33, 26.6, 204/182.8

**B. FIELDS SEARCHED**

Electronic data bases consulted (Name of data base and where practicable terms used):

APS, BIOSIS, MEDLINE, EMBASE, WPIDS, USPATFULL, SCISEARCH, CAPLUS, CANCERLIT, TOXLINE  
SEARCH TERMS: Ausubel, Frederick, Davis, Ronald, Preuss, Daphne, RFLPs, restriction fragment length polymorphisms, allelic variants, biotinylated primers, and fluoresceinated primers, labeled primers, capture probes, solid supports, binding assays, PCR detection of RFLPs, cleavage sites and, restriction enzyme sites